CHAPTER IV

RESULTS AND DISCUSSION

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This chapter presents the results of the five experimental phases and the application of the regression equation evolved from these experiments, to typical vegetarian Indian meals. This is followed by a discussion of the findings of the present study in comparison with other in vitro studies as well as the in vivo ones. The dose effect of the different constitutents selected and their interactions, in the pure system and the standard meal, are analysed in the light of available literature on the mechanism of action of these constituents which provides an evaluation of the in vitro procedure. The use and application of the equation are then discussed and the advantages and limitations are explored. The discussion proceeds on the tacit understanding that the in vitro availability has certain inherent limitations because it does not take into account the mucosal and humoral factors that determine iron absorption in vivo.

RESULTS

Phase I

Validation of the in vitro method

In the first phase, an attempt was made to validate further the in vitro technique used for estimating iron availability in the present study. This was carried out by comparing the in vivo, human iron absorption values from 12 selected meals as reported in the literature, which ranged from 0.8 to 38.8%, vis-a-vis the estimated in vitro iron availability values for the same meals.

Nutrient composition of meals : Table 19 indicates the nutrient composition of the 12 selected meals as analysed in the laboratory. Since there were few reported values for various nutrients in the respective studies, the analysed values were compared with the food table calculated ones. It was evident that for all the nutrients shown in Table 19, the analysed values were lower than the calculated ones. In general, the estimated values for fat and protein ranged from 70 to 90% of the calculated values for these constituents. Ascorbic acid which is known to be decreased after cooking, was lowered by 46 to 55% in the cooked meals that originally contained moderate to high ascorbic acid (11 to 75 mg). In other meals which had a low native ascorbate content (<9 mg), there was almost 85 to 100% difference between the analysed vs calculated values. Similarly, values for tannate were 45 to 67% of the calculated ones while for phytin phosphorus, the difference was of a greater magnitude; the analysed values being a mere 8 to 13% of the calculated values for this constituent. Calcium and phosphate values were 56 to 75% of the calculated values while oxalate showed a wider variation, the analysed values ranging from 27 to 97% of the calculated ones. The reasons which could possibly explain some of these difference are discussed later in the chapter.

Table 20 indicates the total, soluble and available iron content, as estimated for the 12 meals in the present study. The values for total iron and in vivo absorption of iron (%) as reported by the respective authors are also presented in the same table. In contrast to the other nutrients discussed earlier, the

TABLE 19

Nutrient Composition of the 12 selected meals, chemically analysed vs calculated values (Phase I) (mean ± SE)

Meal No.	Fat (g)	Protein (g)	Ascorbic acid (mg)	Tannate (mg)	Phytin Phosph-orus (mg)	Calcium (mg)	Phosph-orus (mg)	Oxalate (mg)
1	19.7±0.16 (24.5)	9.1±0.01 (11.1)	0.4±0.01 (10.1)	127±1.7 (240)	0 (7)	98±0.9 (129)	246±1.5 (328)	8.0±0.01 (10.1)
2	22.8±0.17 (25.8)	26.5±0.09 (29.3)	3.2±0.03 (21.5)	0 (67.5)	29.5±0.56 (229)	268±1.8 (406)	565±1.9 (685)	31.8±0.10 (32.9)
3	14.5±0.09 (25.1)	9.4±0.01 (11.4)	0 (9.4)	411±1.2 (649)	31.7±0.41 (255)	311±1.1 (513)	268±2.4 (423)	0 (9.7)
4	21.4±0.07 (27.9)	11.7±0.03 (15.1)	0 (9.4)	118±0.9 (217)	25±0.09 (211)	98±1.3 (130)	258±1.9 (350)	5.7±0.01 (21.7)
5	8.5±0.14 (11.9)	23.1±0.15 (27.6)	75.6±0.80 (160.2)	139±0.5 (250)	0 (31)	119±1.0 (187)	193±2.5 (347)	21.4±0.53 (48.9)
6	2.6±0.01 (3.3)	16.7±0.01 (19.6)	0 (0)	0 (0)	47.1±0.35 (363)	23±1.0 (30.6)	294±1.9 (492)	0 (6.3)
7	3.6±0.04 (4.4)	20.3±0.03 (22.3)	11.6±0.23 (11.4)	161±1.4 (276)	25.5±0.16 (228)	38±0.9 (65.9)	307±1.9 (456)	226±0.72 (269)
8	10.8±0.15 (14.4)	26.7±0.02 (30.9)	13.0±0.10 (11.6)	137±0.7 (286)	23.7±0.56 (228)	60±0.8 (710)	397±2.4 (550)	236±0.17 (269)
9	28.8±0.20 (34.3)	13.3±0.17 (19.3)	0 (0)	0 (0)	9.2±0.05 (122)	354±2.7 (592)	358±4.5 (553)	0 (2.4)
10	2.7±0.11 (3.7)	18.7±0.02 (21.8)	18.1±0.02 (40.7)	79±0.6 (206)	5.3±0.03 (161)	57±0.7 (84.7)	390±1.5 (558)	15.2±0.09 (26.7)
11	1.8±0.01 (2.0)	15.0±0.02 (17.9)	20.5±0.10 (37.0)	35±0.3 (147)	5.6±0.31 (140)	36±1.6 (62.8)	229±1.8 (329)	22.0±0.03 (31.1)
12	8.3±0.01 (10.6)	20.1±0.01 (22.7)	39.0±0.09 (80.6)	83±0.1 (186)	5.8±0.12 (136)	165±0.7 (255)	311±1.1 (453)	6.0±0.14 (23.3)

1 Each value represents mean of four replicates

2 Figures in parentheses refer to the values, calculated, using the food tables (Gopalan et al, 1989; Diem and Lentner, 1970; Gillooly et al, 1983 and Narasinga Rao and Prabhavati, 1982).

TABLE 20

Total, soluble, ionisable and in vitro available iron as estimated[1] for 12 selected meals vs the values reported[2] by respective authors (Phase I)

In vivo iron absorption (%)	In vitro available iron[3] (%)	Ionisable iron (%) mean±SE	Soluble iron (%) mean±SE	Estimated total iron (mg) mean±SE	Reported total iron[4] (mg)	% difference
0.8	3.3	6.0±0.38	25.5±0.23	6.8±0.04	7.6	-10.5
0.9	2.8	4.9±0.01	20.3±0.30	8.0±0.03	8.3	-3.6
1.3	4.9	9.5±0.05	38.2±0.90	2.9±0.01	2.9	0
1.8	3.1	5.6±0.30	24.6±0.58	5.8±0.05	5.1	13.7
2.0	4.5	8.5±0.82	34.8±0.70	7.9±0.26	7.6	3.9
3.2	3.9	7.2±0.06	21.8±0.90	6.3±0.01	4.7	34
3.9	6.7	13.4±0.74	29.8±0.73	5.5±0.02	5.1	7.8
4.5	8.3	16.7±1.11	57.5±2.51	8.6±0.25	9.6	10.4
6.7	9.2	18.6±0.13	56.0±3.20	3.5±0.01	3.6	-2.7
11.1	11.1	22.5±0.05	68.3±0.83	4.6±0.21	3.7	24
12.0	13.5	27.7±0.85	74.9±0.78	8.9±0.30	7.6	17
38.8	24.2	50.5±0.40	74.1±1.68	6.0±0.03	4.3	39

1 Each value represents mean of four replicates.

2 After calibration to 40% reference dose absorption as reported by the respective authors; arranged in increasing order of iron absorption values.

3 Calculated on the basis of the prediction equation

$$Y = 0.4827 + 0.4707\ X;$$ where Y = % in vitro available iron; X = % ionisable iron

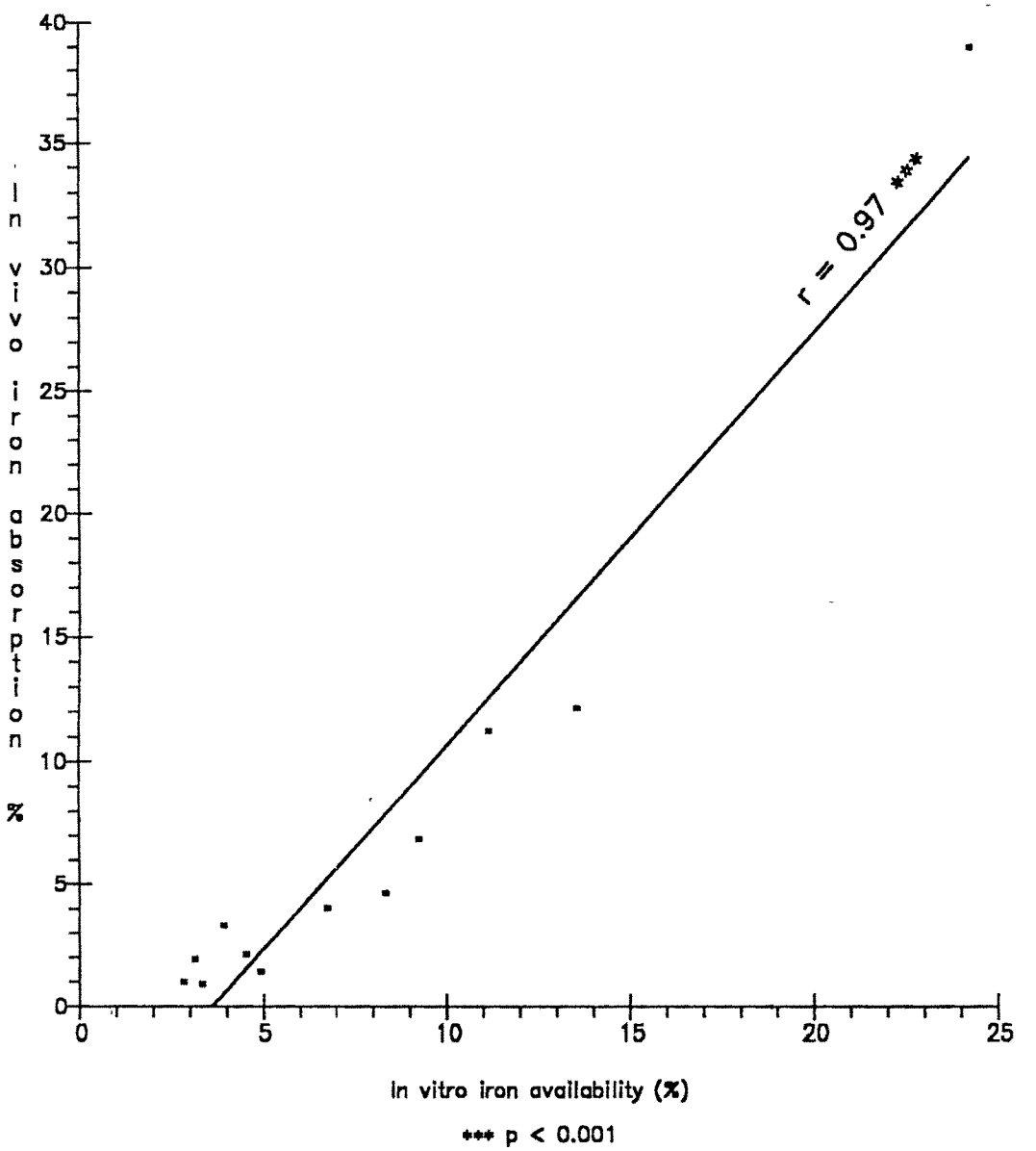
4 These do not include the tracer iron added to the meals.
(Appendix III gives the soluble and ionisable iron in mg ±SE)

total iron values as analysed were generally higher than those reported by the respective authors. Of the twelve meals analysed, seven contained more iron than that reported by the original authors, the difference ranging from 3.9 to 39%, three contained less iron (the differences being 2.7 to 10.5%), while two meals contained the same amount of iron.

Despite these differences in total iron values, the in vitro availability of iron showed a trend that was essentially similar to that observed in the in vivo system. This resulted in a very high correlation (r=0.97) between the two sets, as indicated in Figure 11. This corroborated well with the correlation reported by Narasinga Rao and Prabhavati (1978) between the in vitro iron availability and in vivo iron absorption in humans (r=0.94). The absolute values for iron availability in the in vitro system were higher than those observed in the in vivo system which is a reflection of the fact that the in vitro methods do not mimick the in vivo procedures fully. However, the meals with the lowest iron absorption values in vivo, (i.e. 0.8 and 0.9%) yielded the lowest values for in vitro iron availability (i.e. 3.8 and 2.8% respectively). Similarly for the meals that had the highest iron absorption values in vivo (i.e. 12.8 and 38.8%) the in vitro iron availability was also the highest (i.e 13.4 and 24.2% respectively). The soluble iron values in all instances were higher than the ionisable iron and the trend for soluble iron was similar to that of ionisable iron.

Figure 11

Correlation between analysed in vitro iron availability vis–a–vis in vivo iron absorption from 12 selected meals, as reported by the respective authors (Phase I)



The results of these investigations extended the validity of this particular in vitro technique and therefore this method was used as an indicator of iron availability in all subsequent phases.

Phase II

Dose effect - pure system

The effect of six food constituents, added at five dose levels to the pure system ($FeCl_3$, providing 3 mg elemental iron) on the in vitro iron availability is shown in Table 21 and Figure 12. The availability of iron from $FeCl_3$ solution per se was 21%. Effect of various food constituents on iron availability is presented below.

Ascorbic acid : Addition of increasing doses of ascorbic acid to the pure system resulted in significant dose related increases in available iron until it reached the maximum value of 50% with the dose level 46.5 mg, after which there was no further increase. At this point, the ionisable iron reached 3 mg level, indicating that all the ferric iron in the solution was converted to Fe^{2+} (% ionisable iron = 100). Hence there was no scope for further enhancement in the in vitro iron availability, which was calculated on the basis of % ionisable iron. The molar ratio of ascorbic acid to iron that resulted in such enhancement was 5:1 (46.5 mg ascorbic acid: 3 mg iron). As can be seen from Figure 12, the increase in iron availability was greater at lower levels of ascorbic acid and became increasingly smaller at higher levels. Thus in the pure system a molar ratio of ascorbic acid

TABLE 21

Dose effect of various enhancers and inhibitors on ionisable and in vitro available iron from the pure system[1] (Phase II)

Variable and dose level		Ionisable iron		In vitro available iron	
		mg (mean±SE)	% (mean±SE)	mg	%
Pure system per se		1.29±0.01	43±0.5	0.63	21
Pure system + Ascorbic acid mg (molar ratio to iron)					
2.8	(0.3)	2.10±0.04	70±0.7	1.00	33
5.6	(0.6)	2.18±0.03	72±0.8	1.02	34
9.3	(1.0)	2.47±0.05	82±0.9	1.17	39
23.2	(2.5)	2.85±0.03	95±1.0	1.35	45
46.5	(5.0)	3.00±0.02	100±0.9	1.44	48
186.0	(20.0)	3.00±0.03	100±0.7	1.44	48
Pure system + Citric acid mg (molar ratio to iron)					
30	(3)	1.29±0.01	43±0.3	0.63	21
240	(24)	1.29±0.01	43±0.3	0.63	21
480	(48)	1.29±0.02	43±0.5	0.63	21
720	(72)	1.29±0.01	43±0.3	0.63	21
960	(96)	1.29±0.02	43±0.5	0.63	21
Pure system + Tannate mg (molar ratio to iron)					
25	(0.3)	1.90±0.03	63±0.8	0.90	30
100	(1.0)	1.24±0.03	41±0.9	0.60	20
200	(2.1)	0.95±0.01	31±0.7	0.45	15

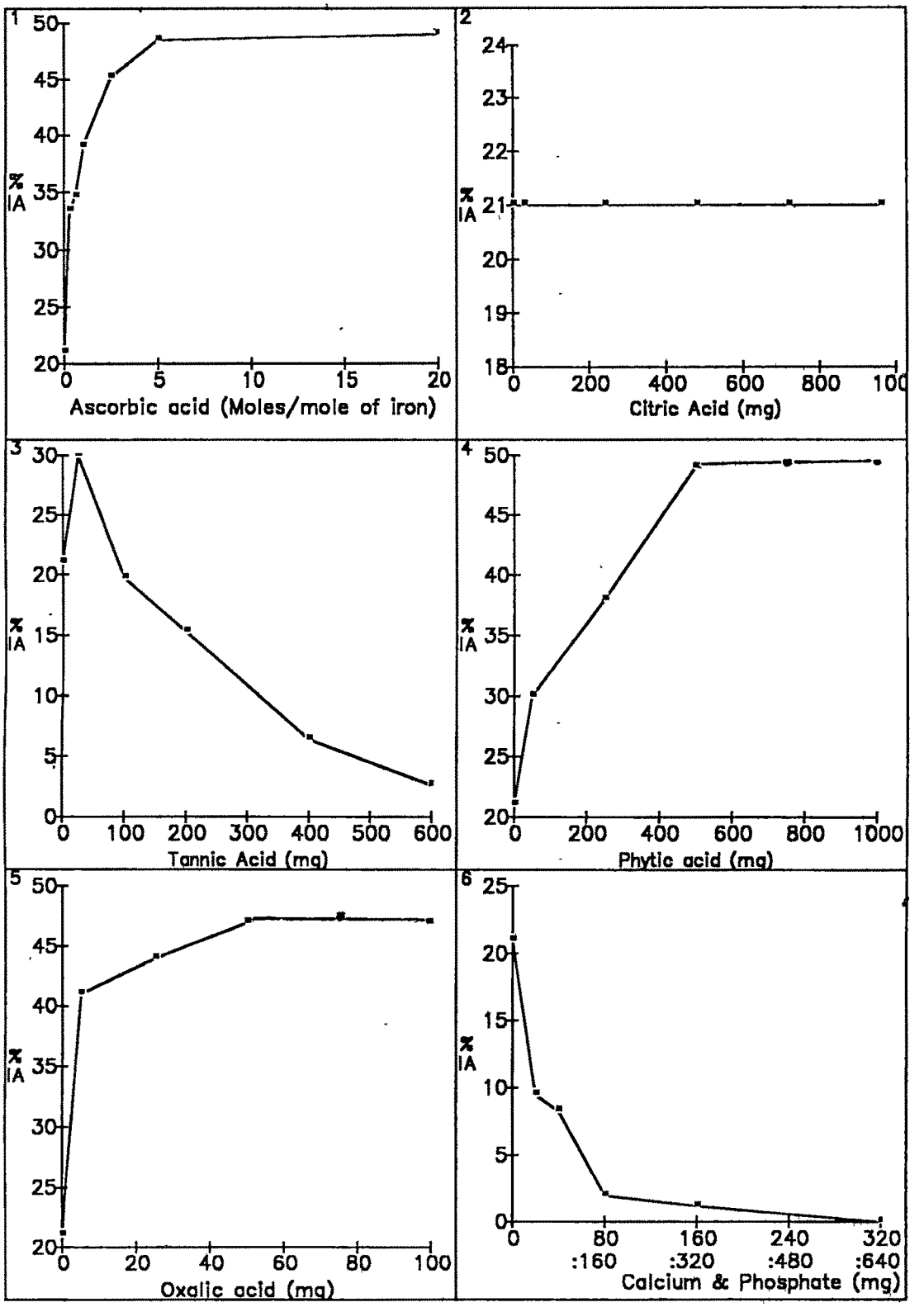
Table 21 contd...

400	(4.3)	0.38±0.01	12±0.6	0.18	6
600	(6.5)	0.14±0.01	4±0.3	0.07	2
Pure system + Phytate mg (molar ratio to iron)					
50	(1.4)	1.9±0.01	63±0.7	0.90	30
250	(7.1)	2.4±0.10	80±0.9	1.14	38
500	(14.3)	3.0±0.20	100±1.1	1.44	48
750	(21.4)	3.0±0.11	100±1.0	1.44	48
1000	(28.6)	3.0±0.13	100±1.1	1.44	48
Pure system + Oxalate mg (molar ratio to iron)					
5	(1)	2.6±0.05	86±0.7	1.23	41
25	(5)	2.8±0.07	93±0.9	1.32	44
50	(10)	3.0±0.04	100±0.7	1.44	48
75	(15)	3.0±0.05	100±0.9	1.44	48
100	(20)	3.0±0.04	100±0.8	1.44	48
Pure system + Calcium phosphate mg (molar ratio to iron)					
20:40	(9:24)	0.58±0.01	19±0.1	0.28	9.5
40:80	(19:48)	0.50±0.01	16±0.1	0.25	8.3
80:160	(37:97)	0.10±0.01	3±0.1	0.06	2.0
160:320	(75:193)	0.05±0.01	1±0.1	0.03	1.2
320:640	(150:387)	0.00	0	0.00	0.0

1 Each value represents mean of four replicates. The iron content of the pure system was 3 mg/250 ml.

Figure 12

Dose relationship of enhancers and inhibitors with % iron availability (% IA) from pure system (Phase II)



to iron of 5:1 was sufficient to convert all of the iron into ionisable form (100%)

Citrate : When added alone at five dose levels to the pure system, citrate failed to show any enhancing effect on iron availability.

Tannate : Among the inhibitors, tannate exhibited marked dose dependent inhibition of iron availability. With the highest dose level of tannate (600 mg), only 2.3% of the iron was available, indicating that almost all the iron (2.93 mg) in the solution was bound at this dose level. In terms of moles, 6.5 moles of tannate were needed to complex one mole of iron from the pure system in the in vitro situation.

Calcium phosphate : Similar to tannate, addition of calcium phosphate also resulted in highly significant dose dependent reduction in iron availability, binding all 3 mg of iron at the highest dose level (320 mg: 640 mg, calcium and phosphate respectively). Iron availability at this point was zero. (Table 21 and Figure 12)

Phytate and Oxalate : These two food constituents that were believed to have an inhibitory effect on iron availability, however, resulted in an unexpected increase in ionisable iron, thus resulting in progressive increase in in vitro available iron.

Phase III

Interaction effect - pure system

The ionisable iron and the in vitro available iron were estimated in the pure system with the addition of all possible combimations of two levels of the six dietary constituents identified for this study. These data were then subjected to multiple correlation and subsequent regression analysis based on which, the prediction equation (no.1) was evolved. Table 22 shows the multiple correlation of each of the six variables with iron availability and the regression analysis carried out on the data. The regression equation evolved from the pure system and the % accountability in variation by the six variables is shown in Figure 13.

Ascorbic acid : Of the six factors, ascorbic acid showed a significant positive correlation ($r=0.375$; $p<0.01$) and accounted for 13% of the variation in iron availability, thereby emerging as a strong enhancer.

Citrate : A new and interesting finding was that citrate, which had failed to show any enhancing effect when added alone to the pure system, exhibited a pronounced enhancing effect on iron availability, when added in combination with other enhancers and inhibitors. It showed an almost equal correlation coefficient ($r=o.367$; $p<0.01$) as that observed with ascorbic acid; also accounting for the same amount of variability (13%) in iron availability. These findings indicated that citrate, in

TABLE 22

Correlation coefficients and regression analysis of the interaction effect of various enhancers and inhibitors on % iron availability from the pure system (Phase III)

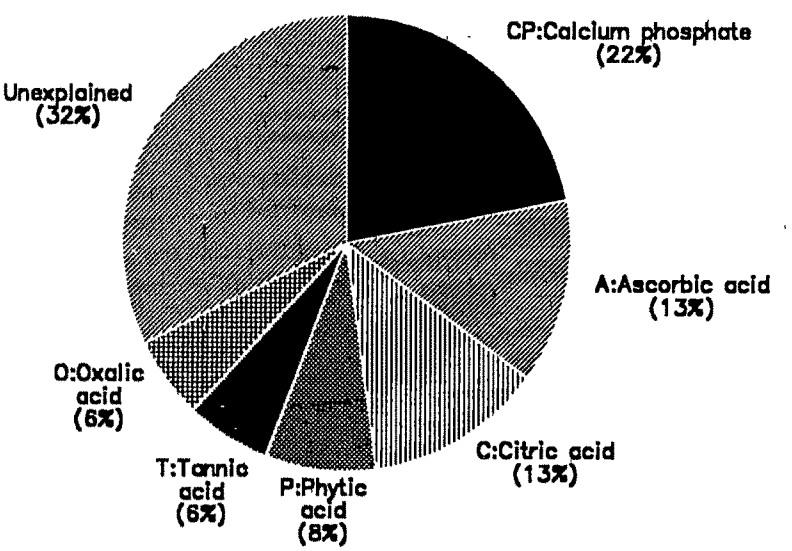
Variable	Correlation coefficient	Adjusted R square	Cummulative adjusted R square	't' values for the regression coefficients
Ascorbic acid	+ 0.375 **	0.13	0.13	0.0000
Citric acid	+ 0.367**	0.13	0.26	0.0000
Tannic acid	- 0.255 *	0.06	0.32	0.0006
Phytic acid	+ 0.283 *	0.08	0.40	0.0002
Oxalic acid	+ 0.251 *	0.06	0.46	0.0007
Calcium phosphate	- 0.483 ***	0.22	0.68	0.0000

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure 13

Regression analysis of the interaction effect of various enhancers and inhibitors on % iron availability from the pure system (Phase III).

$$Y\ (\%\ \text{Iron availability}) = 14 - 0.037\ CP + 0.202\ A + 0.009\ C + 0.007\ P - 0.012\ T + 0.062\ O$$



combination with ascorbate, could prove to be a promising enhancer of iron availability.

Tannate and calcium phosphate : Both tannate and calcium phosphate showed significant negative correlation ($r=-0.255$; $p<0.05$ and -0.483; $p<0.001$ respectively) with iron availability. Calcium phosphate, in the ratio of 1:2 was more inhibitory accounting for 22% of the variation, than tannate which accounted for only 6% of the variation in iron availability.

Phytate and oxalate : These two constituents continued to behave differently than what was expected on the basis of the in vivo studies in the literature. Both demonstrated a positive correlation, together accounting for 14% of the variability. Nearly one third (32%) of the variability in iron availability remained unexplained by the six food constituents selected for investigations in the present study.

As the study progressed it became evident that the equation developed on the basis of the pure system had some serious limitations in prediciting iron availability from the complex meal system, as discussed in a later section. Hence parallel experiments on dose effect and interaction effects were carried out in standard cereal meal system (STD meal), the results of which are presented below.

Phase IV

Dose effect - STD meal

Table 23 and Figure 14 show the dose effect of various enhancers and inhibitors on the in vitro iron availability from

TABLE 23

Dose effect of various enhancers and inhibitors on soluble, ionisable and in vitro available iron from the standard meal (Phase IV)

Variable and dose level	Soluble iron		Ionisable iron		In vitro available iron	
	mg	%	mg	%	mg	%
	(mean ± SE)					
STD meal alone	1.10 ±0.01	37.0 ±0.56	0.28 ±0.01	9.3 ±0.30	0.14	4.8
STD meal + Ascorbic acid mg (molar ratio to iron)						
2.8 (0.3)	1.17 ±0.04	39.2 ±1.53	0.47 ±0.02	15.7 ±0.65	0.23	7.8
5.6 (0.6)	1.30 ±0.06	43.5 ±2.08	0.64 ±0.02	21.5 ±0.80	0.30	10.6
23.2 (2.5)	1.63 ±0.06	54.5 ±2.08	0.66 ±0.03	22.2 ±0.71	0.32	10.9
46.5 (5.0)	1.61 ±0.04	53.8 ±1.60	0.67 ±0.02	22.6 ±0.76	0.33	11.1
186.0 (20.0)	1.90 ±0.08	63.3 ±1.72	0.75 ±0.03	25.1 ±0.78	0.37	12.3
STD meal + Citric acid mg (molar ratio to iron)						
30 (3)	0.75 ±0.02	25.0 ±0.89	0.28 ±0.02	9.5 ±0.63	0.14	4.9
240 (24)	1.30 ±0.04	43.4 ±1.24	0.39 ±0.01	13.2 ±0.50	0.19	6.6
480 (48)	1.33 ±0.03	44.5 ±1.23	0.45 ±0.01	15.2 ±0.30	0.23	7.6
720 (72)	1.38 ±0.08	46.1 ±1.63	0.48 ±0.02	16.2 ±0.50	0.24	8.1
960 (96)	1.31 ±0.08	44.0 ±1.92	0.52 ±0.02	17.3 ±0.53	0.25	8.6

Table 23 contd....

STD meal+ Tannate mg (molar ratio to iron)							
25	(0.37)	1.15 ±0.02	38.3 ±0.89	0.29 ±0.02	9.5 ±0.63	0.15	4.9
100	(1.0)	1.10 ±0.04	37.0 ±1.24	0.28 ±0.01	9.3 ±0.40	0.14	4.8
200	(2.1)	1.00 ±0.02	33.3 ±1.03	0.24 ±0.02	8.1 ±0.36	0.13	4.2
400	(4.3)	0.96 ±0.04	32.0 ±1.54	0.23 ±0.01	7.6 ±0.46	0.12	4.0
600	(6.5)	0.80 ±0.07	26.6 ±2.42	0.21 ±0.01	7.1 ±0.22	0.11	3.8
STD meal + Phytate mg (molar ratio to iron)							
50	(1.4)	0.94 ±0.03	31.3 ±1.49	0.22 ±0.01	7.4 ±0.37	0.12	3.9
250	(7.1)	1.05 ±0.04	35.1 ±1.50	0.25 ±0.01	8.4 ±0.38	0.13	4.4
500	(14.3)	1.06 ±0.04	35.3 ±1.55	0.28 ±0.01	9.5 ±0.39	0.14	4.9
750	(21.4)	1.17 ±0.06	39.0 ±0.87	0.31 ±0.02	10.5 ±0.43	0.16	5.4
1000	(28.6)	1.41 ±0.03	47.1 ±1.24	0.38 ±0.01	12.9 ±0.02	0.19	6.5
STD meal + Oxalate mg (molar ratio to iron)							
5	(1)	1.10 ±0.04	37.0 ±0.95	0.28 ±0.01	9.3 ±0.27	0.14	4.8
25	(15)	1.00 ±0.07	33.3 ±0.87	0.27 ±0.02	9.1 ±0.31	0.14	4.7

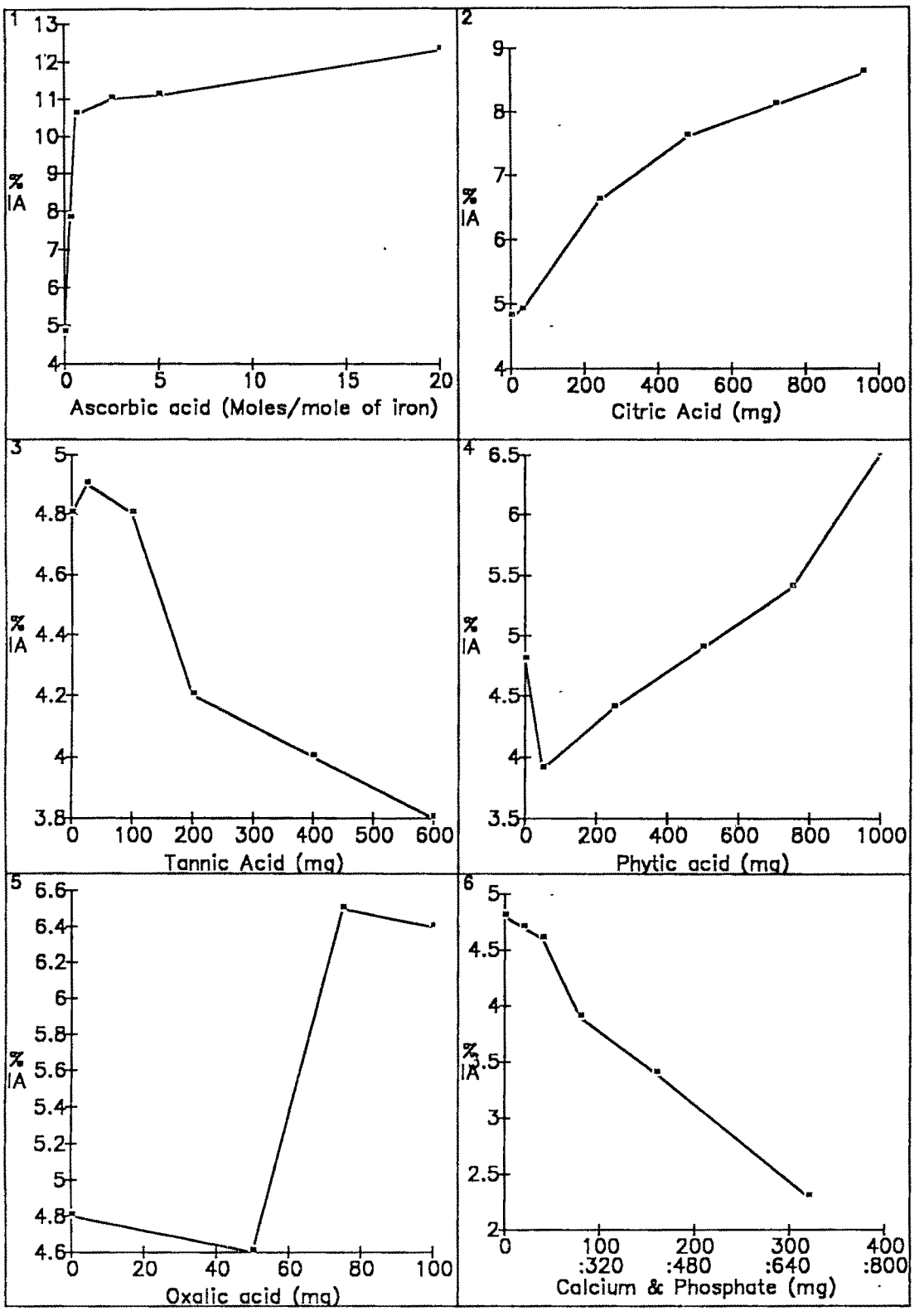
Table 23 contd....

50	(10)	1.00 ±0.02	33.3 ±0.85	0.26 ±0.01	8.8 ±0.61	0.13	4.6
75	(15)	1.48 ±0.06	49.4 ±2.15	0.38 ±0.01	12.8 ±0.41	0.19	6.5
100	(20)	1.48 ±0.06	49.4 ±2.20	0.38 ±0.02	12.6 ±0.70	0.19	6.4
STD meal + Calcium : Phosphate mg (molar ratio to iron)							
20:40	(9:24)	1.08 ±0.03	36.0 ±1.06	0.27 ±0.01	9.1 ±0.38	0.14	4.7
40:80	(19:48)	1.09 ±0.07	36.3 ±2.65	0.26 ±0.01	8.9 ±0.37	0.13	4.6
80:160	(37:97)	1.00 ±0.05	33.3 ±1.26	0.22 ±0.01	7.4 ±0.46	0.12	3.9
160:320	(75:193)	0.80 ±0.03	26.6 ±1.06	0.18 ±0.01	6.2 ±0.41	0.10	3.4
320:640	(150:387)	0.70 ±0.02	23.3 ±0.83	0.12 ±0.01	3.9 ±0.38	0.07	2.3

1. Each value represent mean of four replicates. The total iron content of the STD meal was 3 mg/250 ml of meal homogenate throughout the experiment.

Figure 14

Dose relationship of enhancers and inhibitors with % iron availability (%IA) from standard meal (Phase IV)



the STD meal containing 3 mg of iron. Table 23 also gives the estimated values for soluble iron and ionisable iron. The soluble iron values were in all instances higher than the ionisable iron values, which is consistent with the definition and basis for measurement of these two fractions. Availability of iron from STD meal per se was 4.8%.

Ascorbic acid : Addition of increasing doses of ascorbate brought about a significant increase in iron availability, raising it from 4.8% to a maximum of 12.3%. As against the pure system (Phase II) where all the 3 mg iron could be converted to ionisable iron by addition of 46.5 mg ascorbic acid, in the STD meal, the same amount of ascorbate could convert 1.61 mg of iron to a soluble form,of which only 0.33 mg was in an ionisable (Fe^{2+}) form (Table 23). Interestingly the magnitude of increase in terms of multiples of the basal value with 23.2 mg ascorbic acid was similar in both pure system and STD meal (2.1 times) (21 to 46% and 4.8 to 10.9% respectively). With the increase in ascorbic acid to 46.5 mg, the iron availability increased further only very slightly (45 to 48% and 10.9 to 11.1% respectively). At the highest dose level of 186 mg there was no scope for further increment in iron availability in the pure system, where as in the STD meal the availability increased to 12.3%. These observations indicated that the net increase in iron availability was greater with lower doses of ascorbic acid than that observed with higher doses.

Citrate : In contrast to the pure system, where citrate failed to show any effect on iron availability, in the STD meal, there was a trend of enhancement with increasing levels of citrate, raising the iron availability from 4.8 to 8.6% with the highest dose level (960 mg). Citrate also, at lower levels produced a sharper increase in iron availability (4.8 to 7.6%) and then there was a tendency for the effect to plateu at higher levels (7.6 to 8.6%).

Tannate : Similar to the pure system, tannate significantly reduced iron availability from the STD meal, the effect being clearly dose related. When added at the lower levels (25 to 100 mg), tannate did not produce a noticeable reduction. However, as the level of tannate was progressively increased, there was a concomitant reduction in iron availability until it reached 3.8%. At this point, nearly 2.9 mg of the iron was bound to 600 mg tannate, corresponding to 6.5 moles of tannate per mole of iron, which was surprisingly identical to the molar ratio needed to bind fully, the 3 mg iron in the pure system (PhaseII).

Calcium phosphate : In case of calcium phosphate too, initially there was little reduction in iron availability from the STD meal (4.8% to 4.6% with the first two dose levels) (Figure 14 and Table 23). However, as the amount of these variables increased, it resulted in a significant dose dependent inhibition of iron availability, the highest dose level binding 2.93 mg iron, thereby making most of the iron unavailable

for solubilisation. The in vitro iron availability at this level of Ca and P was only 2.3%. The extent of reduction in iron availability with the addition of each level of calcium and phosphorus in the pure system was very much higher than in the STD meal system.

Phytate and oxalate : Once again, phytate and oxalate resulted in an increase rather than decrease in ionisable iron, thereby raising the iron availability from the STD meal. Figure 14 shows that at the lowest level of added phytate or oxalate there was a tendency for the in vitro available iron to be decreased, though this was small in magnitude. For instance, with the addition of 50 mg of phytate to the STD meal, the in vitro availability dropped from 4.8% to 3.9%. But on raising the phytate level to beyond 500 mg there was an increase in the soluble and ionisable iron fraction resulting in an increase in the in vitro available iron. Similarly, with the addition of 25 and 50 mg of oxalate to the STD meal, the in vitro available iron dropped from 4.8% to 4.7% and 4.6% respectively. When the level of oxalate was raised to 75 mg or more, there was an increase in the available iron to 6.5%.

A sub study under Phase IV

In view of the inconsistent findings observed with the pure, crystalline form of phytate in the preceeding phases, certain experiments were carried out in this phase with bran, dephytinised bran and mono-ferric-phytate (M-F-Phy) to determine the effect of naturally occurring forms of phytate in foods, on iron availability, from a pure system. Wheat bran and rice bran

were both tried. Results of these experiments are indicated in Table 24 and Figure 15. It may be recalled here that wheat bran was added at three levels to correspond to 50, 100, 150 mg of phytate. Wheat bran addition beyond this level could not be done as it raised the level of iron beyond 3 mg. Rice bran, however, by virtue of its lower iron content, could be tried at higher levels to correspond to 200, 250, 500 and 750 mg phytate. Total iron content of the bran samples was estimated and the concentration of iron from $FeCl_3$ solution was adjusted in such a way as to keep the total iron constant at 3 mg/250 ml.

Trials with bran and dephytinised bran: When bran from wheat or rice was added to the pure system in amounts equivalent to various dose levels of phytate (50 to 750 mg) it was found that there was a progressive reduction in iron availability (from 21% to 1.9%), indicating clearly that there was a dose dependent reduction in iron availablity on addition of bran. With addition of wheat bran equivalent to 150 mg of phytate, the iron availablity was reduced by one half. With rice bran equivalent to 500 mg phytate, the availability was reduced to 1/7th and at a bran level equivalent to 750 mg of phytate, the availability was reduced to about 1/10th of the pure system without added bran. Unmistakably, bran in the in vitro system reduced iron availability markedly, keeping in line with the in vivo findings. Dephytinising the bran with dilute HCl resulted in a decrease in the inhibitory effect of bran, although this procedure could overcome the inhibition only partially (91% reduction with bran vs 43% reduction with dephytinised bran).

TABLE 24

Effect of bran (wheat and rice), dephytinised bran or mono-ferric-phytate on % iron availability from the pure system (sub study of Phave IV)

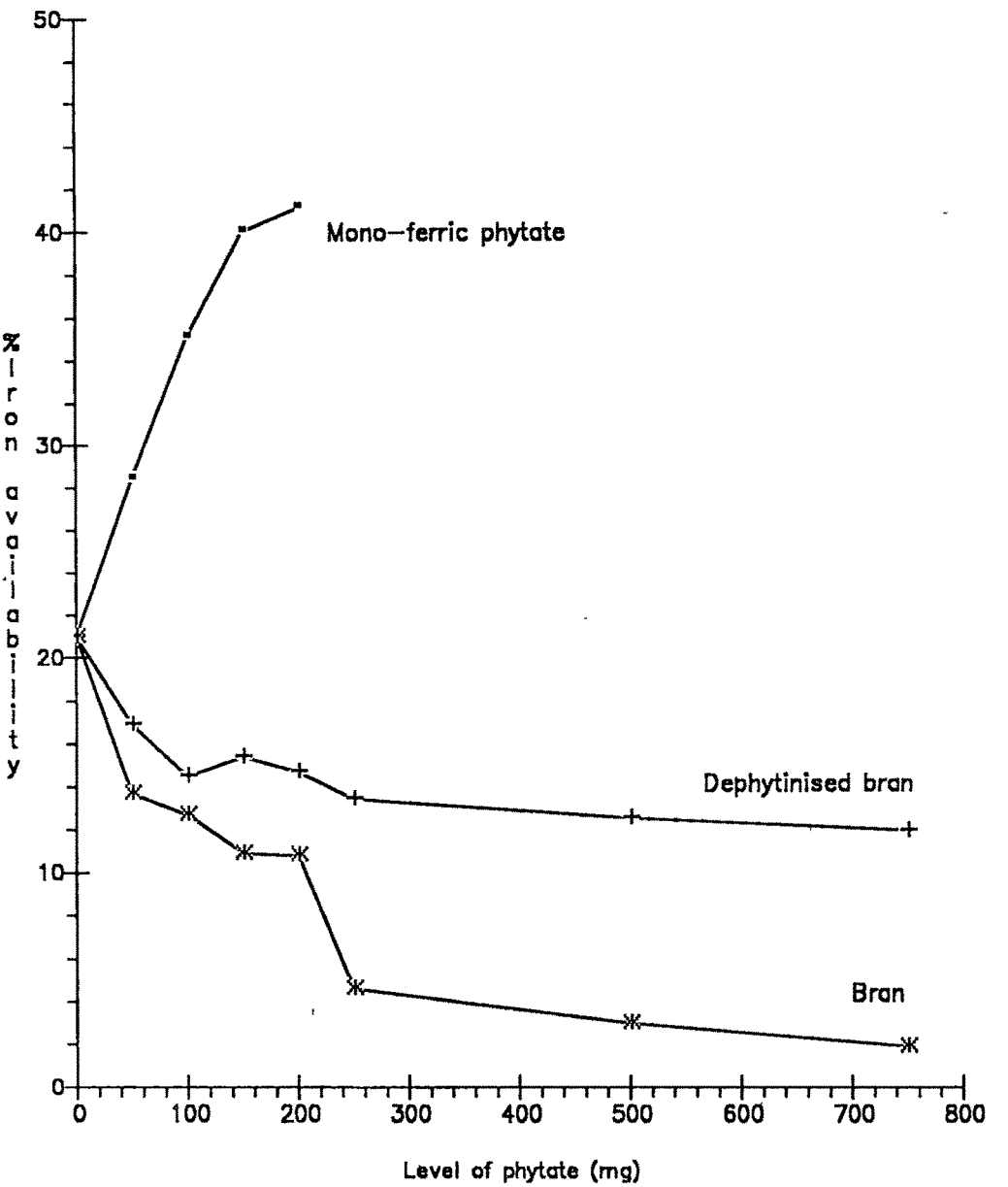
Type of bran (amount equivalent to mg phytate)	Bran		Dephytinised Bran		M-F-Phy+Mg-K-Phy	
	Ionisable iron (mg) (mean ± SE)	% Iron availability	Ionisable iron (mg) (mean ± SE)	% Iron availability	Ionisable iron (mg) (mean ± SE)	% Iron availability
Pure $FeCl_3$	1.32±0.02	21.0	1.32±0.02	21.0	1.32±0.02	21.0
Wheat bran (50)	0.84±0.01	13.7	1.04±0.02	16.9	1.79±0.02	28.4
(100)	0.78±0.02	12.7	0.89±0.01	14.5	2.20±0.03	35.1
(150)	0.67±0.01	10.9	0.95±0.01	15.4	2.51±0.02	40.0
Rice bran (200)	0.65±0.02	10.8	0.91±0.03	14.7	2.59±0.04	41.0
(250)	0.26±0.01	4.6	0.82±0.01	13.4	-	-
(500)	0.16±0.01	3.0	0.77±0.02	12.5	-	-
(750)	0.09±0.02	1.9	0.73±0.01	11.9	-	-

1. Iron availability from the pure system per se was 21% in each experiment. The total iron content of the solution was kept constant to 3 mg Fe/250 ml.

2. Addition of M-F-Phy and Mg-K-Phy could not be carried out for phytate levels > 200 mg as the total iron content of the resultant solution would have increased beyond 3 mg/250 ml.

Figure 15

Effect of bran, dephytinised bran or mono–ferric–phytate on % iron availability from pure system (sub study under Phase IV).



Trials with M-F-Phy : Addition of a complex mixture of M-F-Phy and Mg-K-Phy in amounts equivalent to 50 to 200 mg phytate to the pure system did not show any reduction in iron availability. In fact, the same trend of increased iron availability was observed as that with pure Na-phytate in earlier phases.

These findings taken along with the dose effect of sodium phytate revealed that the in vitro effect of pure, crystalline form of phytate could not be taken as representative of its effect when present endogenously in a natural food form such as bran. In view of the fact that bran could not be used as a source of phytate without substantially increasing the total iron content of the STD meal and that the analysed phytin phosphorus content of most of the cooked, cereal based meals was very low (Phase I), phytate was excluded from investigations in the next phase. For similar reasons oxalate was also excluded from the next set of investigations, i.e, Phase V.

Phase V

Interaction effect - STD meal

In this phase, the interaction effect of only four variables namely ascorbic acid, citric acid, tannate and calcium phosphate was studied. Multiple correlation and the regression analysis applied on the data is shown in Table 25. The regression equation evolved from the STD meal and the % accountability in variation by individual variables is shown in Figure 16.

TABLE 25

Correlation coefficients and regression analysis of the interaction effect of various enhancers and inhibitors on % iron availability from the standard meal (Phase V)

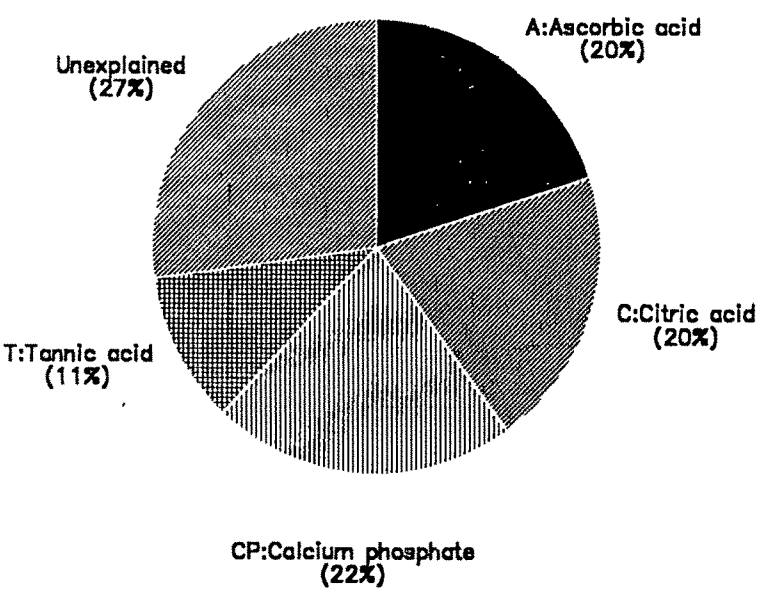
Variable	Correlation coefficient	Adjusted R square	Cummulative adjusted R square	't' values for the regression coefficients
Ascorbic acid	+ 0.477 ***	0.20	0.20	0.0000
Citric acid	+ 0.468 ***	0.20	0.40	0.0000
Calcium Phosphate	- 0.465 ***	0.21	0.61	0.0000
Tannic acid	- 0.325 **	0.11	0.73	0.0015

** $P < 0.01$; *** $P < 0.001$

Figure 16

Regression analysis of the interaction effect of various enhancers and inhibitors on % iron availability from the standard meal (Phase V)

$$Y\ (\%\ \text{iron availability}) = 4.29 + 0.0137\ A + 0.0020\ C - 0.0062\ CP - 0.0026\ T$$



Ascorbic acid : It was observed that the variable to enter the STD meal equation first was ascorbic acid, bearing the highest positive correlation with iron availability ($r=0.477$; $p<0.001$) and accounting for 20% of the variability. Thus, ascorbic acid emerged as the strongest enhancer of iron availability in the STD meal.

Citrate : The second variable to enter the equation was citrate, that exhibited a strong enhancing effect on iron availability ($r=0.468$; $p<0.001$), accounting for another 20% of the variability. This observation with citrate reconfirmed the findings of Phase II and III with the pure system, where citrate alone was not as effective as when present in combination with other enhancers and inhibitors. An encouraging finding in both the systems was that citrate was almost as effective in enhancing iron avaialbility as ascorbic acid.

Calcium phosphate and tannate : The other two variables namely calcium phosphate and tannate entered the equation in that order, as strong inhibitors of iron availability, bearing negative correlations ($r=-0.465$; $p < 0.001$ and $r=-0.325$; $p< 0.001$ respectively). It was evident that calcium phosphate (in the ratio of 1:2) was more inhibitory than tannate in the STD meal system, similar to the findings of the pure system (Phase III). Both these inhibitors together accounted for 33% of the variability in iron availability from the STD meal. Thus, in all 73% of the variation could be explained by these four variables. Nearly one fifth of the variability (27%) still remained unspecified.

Phase VI

The major goal of this phase was to evaluate the predictive powers of the two regression equations evolved from the pure system (Phase III) and the STD meal (Phase V) respectively, when applied to a set of ten typical Indian meals of varying composition. In addition, an attempt was also made to compare the two equations with the only other model available in the literature, evolved by Monsen and Balintfy (1982).

Nutrient composition of the selected Indian meals

Table 26 shows the estimated vs calculated values for total iron and various enhancers and inhibitors contained in the 10 selected typical Indian meals. As can be seen from the Table, the analysed values for all the nutrients were lower than the calculated ones except for iron for which the analysed values were higher than the calculated values. The probable reasons for this kind of difference are discussed later.

Ascorbic acid content of the cooked meals, as analysed, was lowered by 53 to 73% of the calculated values. Tannate too showed 31 to 85% reduction in the analysed values, as compared to the calculated ones. Analysed values for calcium and phosphate were nearly 60 to 80% of the calculated ones. In contrast, phytin-phosphorus content of the cooked meals was only 5 to 20% of its native content in the raw ingredients. Oxalate too varied widely, the estimated values being 16 to 97% of the calculated ones. These results were very similar to the data obtained in

TABLE 26

Total iron content and content of various enhancers and inhibitors in 10 typical Indian meals; actual estimated values vs the calculated values (Phase VI) (mean ± SE)

Meal No.	Nutrient: Total iron (mg)	Ascorbic acid (mg)	Citric acid (mg)	Tannic acid (mg)	Phytin-phosphorous (mg)	Calcium (mg)	Phosphorus (mg)	Oxalate (mg)
1	5.8±0.31 (3.9)	9.4±0.05 (20.1)	(639)	127±1.7 (240)	0 (7)	98±0.9 (129)	246±1.5 (328)	8.0±0.01 (10.1)
2	8.3±0.79 (3.3)	13.0±0.01 (31.5)	(766)	0 (67.5)	29.5±0.56 (229)	268±1.8 (206)	565±1.9 (685)	31.8±0.10 (32.9)
3	8.0±0.03 (5.2)	0 (9.4)	(127)	411±1.2 (649)	31.7±0.41 (255)	311±1.1 (513)	268±2.4 (423)	0 (9.7)
4	6.8±0.04 (5.4)	0 (9.4)	(127)	118±0.9 (217)	25.0±0.09 (210)	98±1.3 (130)	258±1.9 (350)	5.7±0.01 (21.7)
5	5.3±0.07 (3.5)	72.9±0.70 (135)	(0)	31±0.1 (165)	43.5±1.84 (204)	80±1.4 (104)	220±1.3 (305)	7.9±0.48 (15.1)
6	3.8±0.10 (1.6)	0.7±0.01 (2.4)	(156)	0 (0)	8.9±0.19 (104)	26±0.9 (33.1)	182±0.9 (209)	3.2±0.13 (9.2)
7	5.2±0.07 (3.5)	5.2±0.05 (14.6)	(408)	21±0.5 (103)	8.8±0.59 (112)	143±2.1 (185)	203±1.0 (338)	13.7±0.44 (16.0)
8	5.4±0.03 (4.1)	3.0±0.02 (8.5)	(127)	189±1.7 (273)	0 (4.4)	175±1.5 (208)	291±2.8 (361)	7.9±0.20 (10.5)
9	4.0±0.04 (5.1)	21.4±0.63 (26.3)	(186)	26±0.8 (165)	0 (49.8)	113±1.0 (140)	313±2.4 (424)	6.6±0.26 (11.8)
10	3.6±0.05 (2.6)	1.2±0.15 (4.5)	(127)	9±0.2 (57.7)	4.9±0.23 (106)	23±0.5 (36.3)	176±0.6 (267)	8.3±0.27 (10.9)

1 Each value represents mean of four replicates

2 Figures in parentheses refer to the calculated values using the food tables (Gopalan et al, 1989; Diem and Lentner, 1970; Gillooly et al, 1983 and Narasinga Rao and Prabhavati, 1982)

Phase I of the study. The soluble, ionisable and in vitro available iron in the ten meals is laid out in Table 27. The estimated % in vitro availability of iron varied from 1.96 to 8.89 in these meals.

Application of the predictive models

The three predictive equations that were evaluated, as applied to 10 typical Indian meals, are shown in Table 28.

Evaluation of the predictive equations 1 vs 2 : When the analysed values (estimated input) of enhancers and inhibitors were incorporated into the two equations from the present study (equation 1 and 2) to predict iron availability from the ten typical Indian meals, it was observed that the predictive power of equation 2 was much higher (r=0.76) than that of equation 1 (r=0.59) as illustrated in Figure 17. A serious limitation in using the equation from the pure system (i.e. equation no.1) was the gross over estimation of the % of total iron available as indicated in Table 29. These findings indicate that the model generated from the pure system was not useful in predicting the availability of iron from complex meal system, as it yielded values that were 9 to 60 times higher than the in vitro estimated values from the respective meals. Besides, there were also other differences; the extent of reduction in iron availability by tannate and calcium phosphate for similar levels were very different for the pure system vs the STD meal system. On the other hand, the model generated from the standard meal provided a more realistic, quantitative estimate of iron availability from cereal based meals of varying composition.

TABLE 27

Soluble, ionisable and in vitro available iron content of the ten, typical Indian meals[1] (Phase VI)

Meal No.	Soluble Iron		Ionisable Iron		In vitro available iron	
	mg	%	mg	%	mg	%
	←——— (mean ± SE) ———→					
1	2.0±0.01	34.6±0.13	0.91±0.01	15.8±0.22	0.45	7.95
2	3.6±0.05	43.7±0.47	1.40±0.09	17.8±0.12	0.73	8.89
3	1.6±0.01	20.3±0.30	0.39±0.01	4.9±0.01	0.22	2.81
4	1.7±0.01	25.5±0.23	0.41±0.02	6.0±0.38	0.22	3.33
5	0.9±0.01	16.2±0.27	0.58±0.01	11.0±0.17	0.29	5.68
6	0.7±0.21	18.9±0.62	0.48±0.05	12.6±0.32	0.24	6.44
7	1.2±0.15	23.5±0.71	0.71±0.02	13.6±0.64	0.35	6.87
8	0.5±0.02	9.2±0.06	0.17±0.01	3.1±0.10	0.10	1.96
9	1.4±0.31	34.3±0.87	0.67±0.04	16.6±0.25	0.33	8.33
10	0.9±0.14	25.5±0.42	0.57±0.01	15.5±0.06	0.28	7.81

1 Each value represents mean of four replicates.

TABLE 28

The two predictive models evolved in the present study and the model of Monsen and Balintfy (1982), as applied to 10 typical Indian meals (Phase VI).

Equation 1 (based on pure system)

% In vitro iron availability = 20.96[1] - 0.079 calcium phosphate[2] + 0.2022 Ascorbic acid + 0.0093 Citric acid - 0.0120 Tannic acid

Equation 2 (based on STD meal)

% In vitro iron availability = 4.29 + 0.0137 Ascorbic acid + 0.0020 Citric acid - 0.0062 Calcium phosphate[2] - 0.0026 Tannic acid

Equation 3 (Monsen and Balintfy, 1982)[3]

$$\%\ \text{Bioavailable iron} = 3 + 8.93 \log_n \frac{(\%\ \text{BI})}{\frac{EF+100}{100}}$$

where EF=enhancing factors (ascorbic acid, meat, fish, poultry)

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1. In view of the fact that phytate and oxalate were excluded from the STD meal equation, they were also omitted from this equation, which resulted in an increase in the regression constant from 14.0 to 20.9.

2. For predicting iron availability, calcium content of the meals was used with the regression coefficient of calcium phosphate in the above two models. Since calcium and phosphate were added together in a ration of 1:2 while deriving the equations, the regression coefficient for phosphate would be half that for calcium. Hence meal content of any one of the nutrients could be used for computations accordingly.

3. This model is applied when EF <75.

When EF = 0 then % BI = 3
EF $\geq$75, then % BI = 8

This is based on the assumption that the individual has body iron stores of 500 mg.

Figure 17
Correlation between analysed % iron availability (%IA)vs computed % iron availability (%CIA) using equation 1 and 2, incorporating analysed (A) or food table values (B) of enhancers and inhibitors.

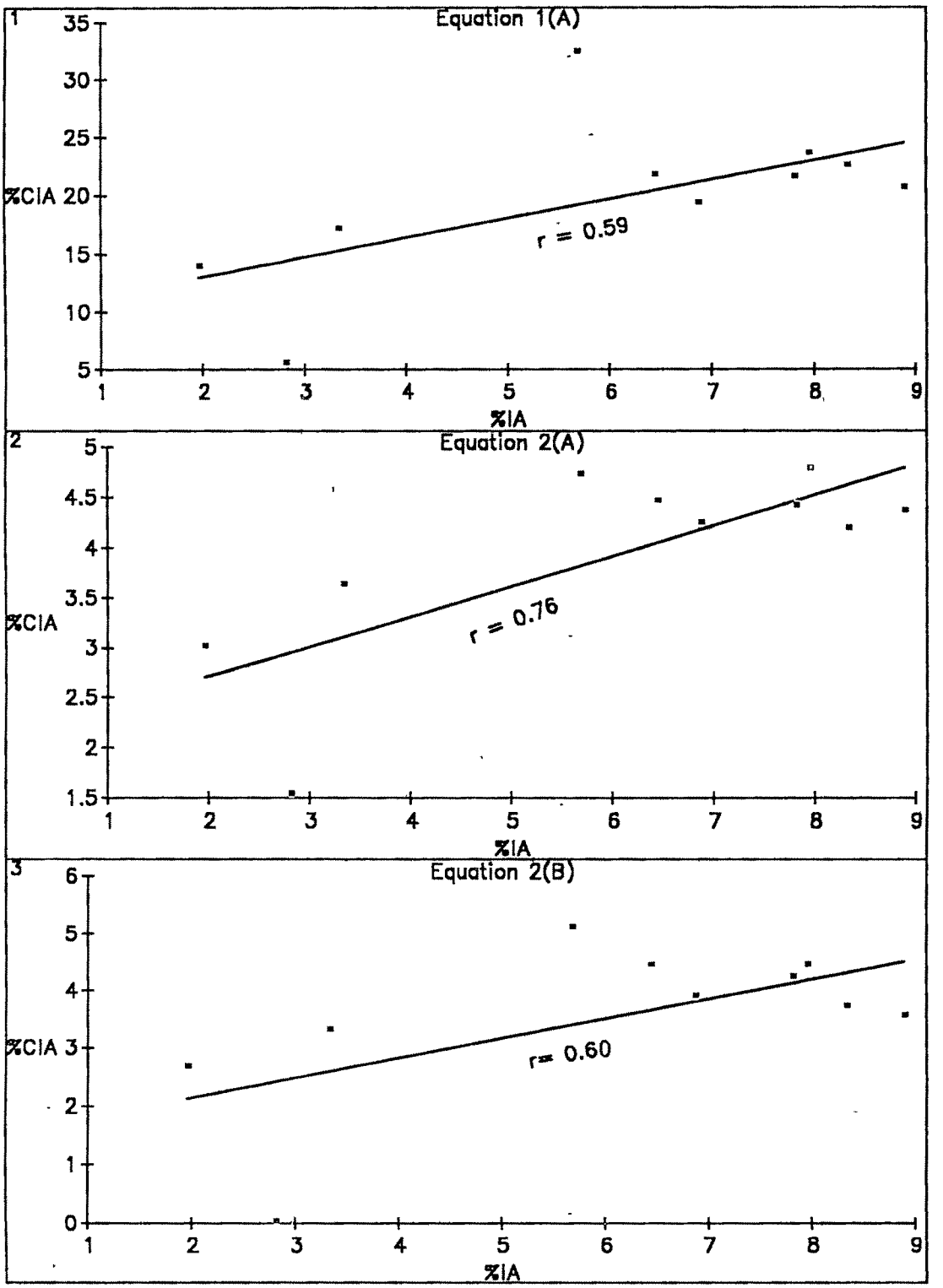


TABLE 29

Estimated % in vitro available iron vs computed % available iron using equation no.1 and 2 as applied to the 10 Indian meals (Phase VI)

Meal No.	Estimated % in vitro iron availability	Computed % available Iron		
		Estimated Input[1]		Calculated Input[2]
		Eqn. No.1	Eqn. No.2	Eqn.No.2
1	7.95	23.57	4.77	4.44
2	8.89	20.55	4.35	3.56
3	2.81	5.38	1.52	0.00
4	3.33	17.01	3.62	3.29
5	5.68	32.30	4.71	5.09
6	6.44	21.59	4.45	4.43
7	6.87	19.25	4.23	3.89
8	1.96	13.80	3.00	2.65
9	8.33	22.40	4.18	3.72
10	7.81	21.42	4.40	4.23

1 Actual estimated values of the enhancers and inhibitors were used for computation.

2 Food table calculated values of the enhancers and inhibitors were used for computation.

Evaluation of equation 2 using food table values of enhancers and inhibitors : When an attempt was made to predict iron availabiliity by incorporating food table values of enhancers and inhibitors in equation 2, computations revealed that there was a correlation coefficient of r=0.60, between the analysed and the computed values for iron availability. This observation indicates that in the absence of analysed values for various enhancers and inhibitors in a meal, food table values can also be used to provide an estimate of available iron, by applying the predictive model (STD meal) evolved in the present study; such a prediction, however is not as good as that obtained when actual analysed values of the enhancers and inhibitors are used (r=0.60 vs r=0.76 respectively).

Evaluation of the present model (equation 2) in comparison with the model of Monsen and Balintfy (1982) (equation 3) : When an attempt was made to evaluate the predictive power of the model that incorporated only enhancers of iron availability (Monsen and Balintfy, 1982), it was observed that when applied to typical Indian vegetarian meals (Table 30), the correlation coefficient obtained between the analysed % iron availability values and the computed % bioavailable iron was very low (r=0.19) as illustrated in Figure 18. This indicates that the present model which incorporates both enhancers and inhibitors of iron availability is more applicable to the Indian meal situation, where inhibitors clearly outnumber the enhancers.

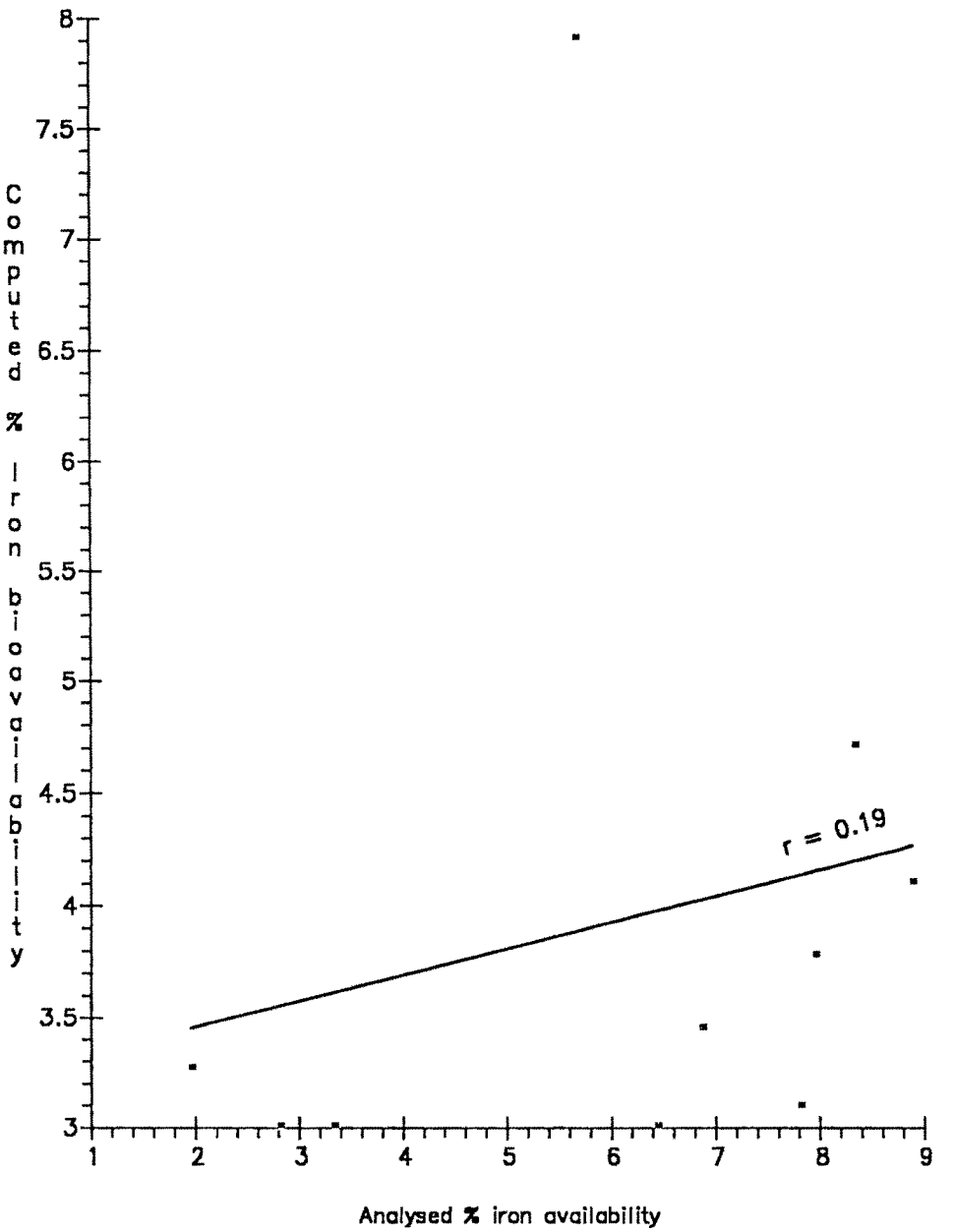
TABLE 30

Estimated % in vitro available iron vs computed % bioavailable iron using equation no.3 (Monsen and Balintfy '82), as applied on 10 Indian meals (Phase VI)

Meal No.	Estimated % in vitro available iron	Computed % available iron using Eqn. No.3
1	7.95	3.77
2	8.89	4.09
3	2.81	3.00
4	3.33	3.00
5	5.68	7.90
6	6.44	3.00
7	6.87	3.44
8	1.96	3.26
9	8.33	4.70
10	7.81	3.09

Figure 18

Correlation between the analysed in vitro iron availability vs bioavailability of iron, computed from the model of Monsen and Balintfy (1982)



DISCUSSION

Nutrient composition of the meals (Phase I and Phase VI)

In the first and the last phase of the study, a number of selected meals were chemically analysed for various nutrients. Findings revealed that for all the nutrients except iron, the analysed values were lower than those calculated using the standard food tables. The difference between estimated and calculated values was much smaller for fat, protein, calcium and phosphorus whereas for the other nutrients the differences were quite large. The reasons for this can be many. Firstly, the varietal differences and seasonal variation can account for atleast some of the differences. Secondly, loss of certain nutrients during preliminary processing and cooking can result in lower estimated values for at least some of the nutrients. This is particularly true for water soluble vitamins such as ascorbic acid, for which upto 60% loss on cooking has been reported (Diem and Lentner, 1970). In the present study, losses on cooking in ascorbic acid varied from 46 to 100%. Hallberg et al (1982) have reported a near total loss of ascorbic acid when foods were held at a high temperature (75^{o}C) for 1 to 4 hrs. They further observed that more than 50% loss in ascorbic acid content of the foods occured in the first hour. In the present study, though the time taken for cooking for the entire meals rearely exceeded one hour, the temperature at which the foods were cooked was however high (boiling, steaming or frying temperature), hence resulting in substantial loss of ascorbic acid on cooking.

In the case of tannates and other polyphenolic compounds, the food table values were 33 to 55% higher than the actual estimated values. This may be because most of the tannis are reported to be present in the outer layers of the cereals, grains and legumes and peels of vegetables and fruits (Narasinga Rao and Prabhavati, 1982; Gillooly et al, 1983). Further, cooking of certain foods such as pulses has been reported to bring about a large decrease in their tannin content (Rao and Deosthale, 1982). The above authors investigated the tannin content of raw vs cooked pulses such as pigeonpea, chickpea, blackgram and greengram. It was observed that on cooking the pulses for 15 minutes under pressure (15 lb), tannin content reduced by 59% in pigeonpea (from 1141 to 475 mg/100 g) and by about 70% in the other three legumes. Pawar and Parlikar (1990) have also reported a 70% reduction in polyphenolic content of pearl millet that was dehulled and soaked in dilute acid (0.2N HCl) for 20 to 45 minutes. In the present study, preliminary steps before cooking such as peeling of vegetables and fruits, use of dehulled and decorticated grains and pulses and the actual process of cooking which required soaking in acidic tamarind juice or addition of lime juice in some instances could have contributed towards lowering of the tannin content of the meals.

Another analysed nutrient which differed greatly from the calculated values was phytin-phosphorus. Though the food table values for phytin-phosphorus were appreciably high for most of the meals, on actual analysis of the cooked meals, it was observed that on an average, only about 10% of the calculated

phytin-P content was recovered from the meals after cooking. In order to cross check this observation, known quantities of pure Na-phytate .were added to the meals and they were analysed for phytin-P. Findings revealed that there was 90 to 95% recovery of the pure Na-phytate added to the meals. Further, the bran samples used in the sub study of Phase IV were also analysed for phytin-P, which yielded values close to the ones reported in Food Tables for bran. The analysed phytin-P value for wheat bran was 195 mg/100g and that for rice bran was 190mg/100g as against 200mg phytin-P reported for bran (100g) in the food tables (Gopalan et al, 1989). This indicated that the method used was fairly accurate and the cooked meals were indeed very low in phytate. Loss of phytate on baking has been repeatedly reported by Hallberg et al (1989 and 1991). For example, the baked wheat rolls used in their studies had a phytin-P content of only 3.7 to 6.0 mg as against 11.6 to 14.2 mg phytin-P contained in the raw wheat flour used for making the rolls. It is possible that during the process of baking or cooking, there may be some degradation of the hexaphosphate moeity, resulting in lower molecular forms such as tri- or tetra-phosphates, thereby lowering the content of precipitable phytate (Sandberg et al, 1988). Yet another reason for the low values for phytin-P in the present study could be the use of sifted wheat flour for cooking. This removed some of the bran fraction from the flour which is known to be rich in phytate. While many studies have reported on the phytin-P content of raw foods, there are few values available on cooked foods, especially processed by traditional Indian

methods of cooking, hence making it difficult to compare the analysed values obtained for phytin-P in cooked meals in the present study.

Another nutrient for which the analysed values were lower than the calculated values was oxalate, the values ranging from 16 to 97% of the calculated values. In a recent study where oxalate content of selected foods (raw) was analysed by Meena et al (1987), it was found that in most of the foods (amaranth, horsegram, sesame and almonds), the analysed oxalate content was lower by 12 to 43% than that reported in the food tables.

The only nutrient which exhibited a higher value on analysis than the food table values was total iron. The total iron values for some meals were markedly higher (on an average, 30%) as compared to the reported values. The probable reason for this could be presence of contamination iron in the ingredients purchased locally. For example in one of the meals (meal no.5, Phase I), commercially prepared hamburger was used for analysis while in others (meal no.8, 10, 11, and 12, Phase I), meat or fish was purchased from local market to be incorporated in the meals. The containment iron could have entered these foods through the use of iron utensils for cooking of meat patties and by the use of iron knives for cutting the fish or meat. It has been reported by Narasinga Rao and Prabhavati (1981) that cereals and pulses, as purchased from the local market, contain at least 20 to 30% of the total iron content as contamination iron. The meals used in Phase VI incorporated locally purchased grains such

as wheat, rice, ragi and sorghum. When these were used for cooking, due to surface contamination, the iron content of these meals could have increased appreciably (32 to 53%) as compared to the food table values.

In vitro availability of iron vs in vivo iron absorption -Phase I

Studies in Phase I, where the in vitro availability of 12 meals, selected from in vivo studies reported in the literature, was compared with the in vivo iron absorption values reported for these, established that there was a high correlation between the two sets (r=0.97). The meals were chosen consciously to include a wide range of iron absorption values ranging from 0.8 to 38.8%. This was done in view of the fact that the correlation coefficient, (r=0.94) as reported by Narasinga Rao and Prabhavati (1978) for the same method was based on a narrow range of iron absorption values (1.6 to 3.8%). The results of this phase indicated that under conditions of a wide range of in vivo iron absorption values also the method yielded a high correlation. The method appeard to be sensitive to both low and high iron bioavailability meals comprising of vegetable foods as well as non-vegetarian foods comprising of heme iron. For example, in the 12 meals selected, the one which had the highest iron absorption in vivo (38.8%) contained beef in the form of a hamburger. The in vitro iron availability for this meal was also the highest (24.2%). Similarly, meals that had the lowest iron absorption values (0.8 and 0.9%) were based on cereals such as ragi and sorghum. These meals resulted in the lowest in vitro iron

availability (2.8 and 3.3%). Thus the validity of this method was extended further to cover a wide range of in vivo iron absorption values and was considered appropriate for use in the other phases of the study.

Effect of enhancers and inhibitors on iron availability (Phase II, III, IV and V) - Dose and interaction effects

The effect of the six dietary constituents i.e. the two enhancers (ascorbic acid and citric acid) and four inhibitors (tannate, phytate, oxalate and calcium phosphate) was investigated on iron availability in the pure system (Phase II) and the STD meal (Phase IV) with two fold objective, a) to establish the trend of dose effects in the vitro situtation, and to compare it with the in vivo findings, and b) to select the levels for studies on interaction effects (Phase IIIand V).

Initial experiments used a pure solution of ferric chloride, providing 3 mg elemental iron as the source of iron. It was observed that the in vitro iron availability from the pure system per se was 21%. In a study by Narasinga Rao and Prabhavati (1982) where they used the same iron compound ($FeCl_3$), the in vitro iron availability was 10%. However, the concentration of iron used by them was nearly 16 times that the level of iron used in the present study (47.5 mg vs 3 mg respectively). This could possibly explain the difference between the magnitude of iron availability from $FeCl_3$ in the two studies. In a recent study by Forbes et al (1989) it was reported that 60 to 75% of the electrolytic iron from $FeSO_4$ was soluble while only 3 to 4% of ferric phosphate iron was solubilised in an in vitro setting.

The availability of iron from $FeCl_3$ which is less soluble than $FeSO_4$ but more so than ferric phosphate may lie somewhere in between these two limits, corresponding to 21% iron availability, observed in the present study from the pure system per se.

In the later part of the study, parallel experiments were carried out using a complex cereal meal as the source of iron. This was designated as the standard meal (STD meal). It was observed that iron availability from the STD meal per se was 4.8%. This tallies well with the other in vitro studies carried out in our laboratory, which have yielded values of 3.7 to 4.9% iron availability from cereal based meals (Saxena and Seshadri, 1988; Chritian and Seshadri, 1989). Similar values have been reported by Narasinga Rao and Prabhavati (1978) for in vitro iron availability from wheat based and rice based meals (2.5% and 3.2% respectively).

The in vitro value obtained in the present study for the STD meal per se, is also in line with the in vivo iron absorption values reported by several investigators for somewhat similar cereal based meals; 1 to 5% for cereal based Indian meals (Narasinga Rao et al,1983), 1.3 to 4.3% from rice based Sout East Asian meals (Aung-Than-Batu et al, 1976), 1.4 to 3.5% from maize and beans based Venezuelan meals (Layrisse et al, 1974) and 3% from rice based Latin American meals (Hallberg and Rossander, 1984).

The effect of various enhancers and inhibitors on in vitro iron availability in varying doses as observed in the present

study is discussed below in relation to the other in vitro and in vivo studies, reported in the literature.

Ascorbic acid

Dose effect : Ascorbic acid when added at different dose levels significantly increased in vitro iron availability from the pure system as well as the STD meal. However, in the pure system much higher amounts of iron could be converted to ionisable form (95%) with a certain level of ascorbic acid (2.5 moles of ascorbic acid to iron) than in the STD meal system (22% of iron in ionisable form with 2.5 moles of ascorbic acid). In terms of magnitude of enhancement from the basal value, ascorbic acid showed similar increase in the pure system and the STD meal at the lowest level of 2.8mg of ascorbic acid (57% and 62% increase respectively; Table 31). However at the next dose level (5.6mg) the increase in the STD meal over the basal value was much higher (two fold) compared to the pure system (1.6 fold). The difference in the magnitude of increase narrowed down at the higher dose levels of 23.2mg to 186mg of ascorbic acid as shown in Table 31.

The magnitude of increase observed in the STD meal system was essentially similar to that observed in other in vitro studies as indicated in Figure 19. In vitro studies carried out by Hazell and Johnson (1987) have shown that addition of 25 mg ascorbic acid nearly doubled the % iron diffusability (4% to 7.7%) from plain white wheat flour. This is

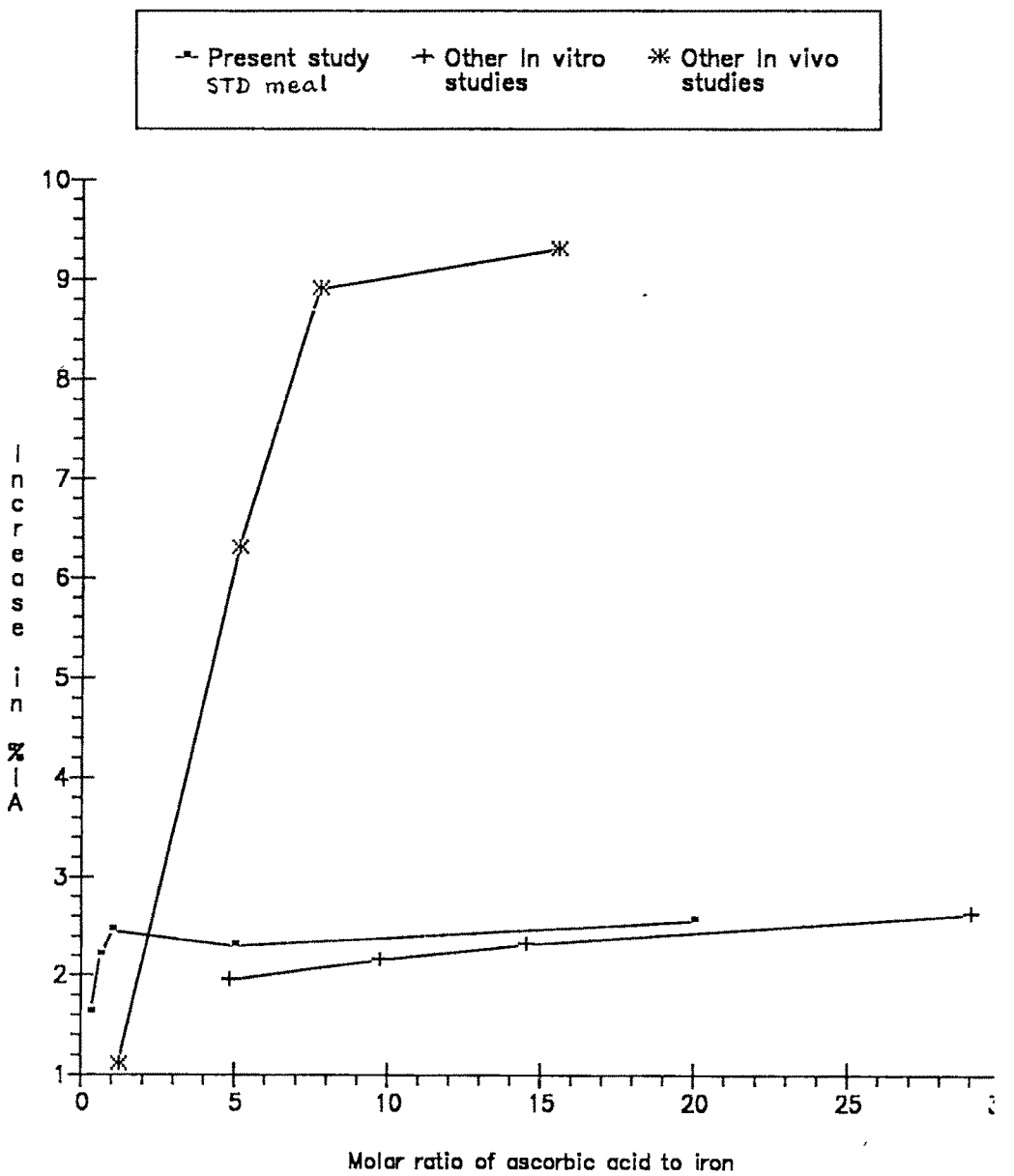
TABLE 31

Magnitude of increase/decrease in iron availability, from the basal value, on addition of increasing doses of the enhancers and inhibitors to the pure system or the STD meal.

Variable and dose level mg (molar ratio to iron)		% Increase (↑) or decrease (↓) in iron availability	
		Pure System	STD meal
Ascorbic acid			
2.8	(0.3)	57 ↑	62 ↑
5.6	(0.6)	62 ↑	121 ↑
23.2	(2.5)	114 ↑	127 ↑
46.5	(5.0)	128 ↑	131 ↑
186.0	(20.0)	128 ↑	156 ↑
Citric acid			
30	(3)	0	2 ↑
240	(24)	0	37 ↑
480	(48)	0	58 ↑
720	(72)	0	69 ↑
960	(96)	0	79 ↑
Tannic acid			
25	(0.3)	43 ↑	2 ↑
100	(1.0)	5 ↓	0
200	(2.1)	28 ↓	12 ↓
400	(4.3)	71 ↓	17 ↓
600	(6.5)	90 ↓	21 ↓
Phytate			
50	(1.4)	43 ↑	19 ↓
250	(7.1)	81 ↑	8 ↓
500	(14.3)	128 ↑	2 ↑
750	(21.4)	128 ↑	12 ↑
1000	(28.6)	128 ↑	35 ↑
Oxalate			
5	(1)	95 ↑	0
25	(5)	109 ↑	2 ↓
50	(10)	128 ↑	4 ↓
75	(15)	128 ↑	35 ↑
100	(20)	128 ↑	33 ↑
Calcium Phosphate			
20:40	(9:24)	55 ↓	2 ↓
40:80	(19:48)	60 ↓	4 ↓
80:160	(37:97)	90 ↓	19 ↓
160:320	(75:183)	94 ↓	29 ↓
320:640	(150:387)	100 ↓	52 ↓

Figure 19

Comparison of dose effect of ascorbic acid on iron availability in the present study with that reported by other in vitro and in vivo studies. (Data taken from table 2A).



similar to the values obtained in the present study, using the STD meal with 23.2 mg ascorbic acid (4.8% to 10.9%).

In vivo, however, ascorbic acid produced a much greater magnitude of enhancement as indicated in Figure 19. For example, five moles of ascorbic acid to iron, resulted in nearly a six fold increase in iron absorption from a low availability maize meal (1.38% to 8%, Layrisse et al, 1974) as against only two and a half fold increase in iron availability in the vitro system in the present study (4.8% to 11.1%). However, if the basal diet in the in vivo system had a relatively higher availability such as 6.3% from a rice, maize, bread and beans meal, the increase in iron absorption on addition of 30 mg ascorbic acid was only three fold (Martinez - Torres et al, 1987). This is closer to the two fold increase brought about by addition of 25 mg ascorbic acid to the STD meal in the present study. As the quantity of ascorbic acid is increased in terms of molar ratio to iron , there is plateuing off of the curve in the in vivo system too. Thus, while the direction of the effect and shape of the curve are similar in the in vitro and in vivo situation, there is a marked difference between the magnitude of effects if the basal meal has a low iron availability value.

Interaction effect : In the present study, when the interaction effect of the selected food constituents was studied (Phase III and V), ascorbic acid emerged as the strongest enhancer of iron availability when added in combination with other enhancers and inhibitors to the pure system or STD meal.

It has been shown in a recent in vitro study carried out in our laboratory (Christian and Seshadri, 1989) that addition of ascorbic acid (100 mg) to a cereal based meal fully counteracted the inhibitory effect of tea (containing 195 mg tannate) on iron availability (Basal meal - 3.9%; meal+tea -2.5%; meal + tea + ascorbic acid - 3.8%).

In the in vivo situation, ascorbic acid was shown to counteract the inhibitory effect of phytin-P (Hallberg et al, 1989). The authors reported that about 80mg ascorbic acid was required to fully counteract the inhibition brought about by 25 mg phytin-phosphorus, in iron absorption from a wheat bun meal. The observation that ascorbic acid emerged as a strong enhancer in the present study, despite the presence of inhibitors such as tannate and calcium phosphate, tallies with the findings of the above in vitro and in vivo studies.

Mechanism of action : The effects of ascorbic acid in the present study are consistent with the mechanism of action proposed for ascorbic acid in the literature (Hurrel, 1984). One mode of action of ascorbic acid is through its ability to act as a strong reducing agent, whereby it converts ferric iron to ferrous iron. The ferric form of iron in an acquous medium tends to exist in a hydrated state and as the pH is raised, the hydrated feric iron is converted to $Fe(OH)_3$ in a process of hydrolysis. As the pH is raised to an alkaline medium the process of hydrolysis becomes irreversible and $Fe(OH)_3$ gets precipitated thus making iron unavailable. Although ferrous

iron also goes through similar reactions, it remains far more soluble at an alkaline pH (10^{-3}M) than ferric iron (10^{-18}M). Thus conversion of ferric iron to ferrous iron by ascorbic acid can be expected to raise the availability of iron substantially and this is dramatically illustrated by the findings in the pure system in the present study. In a pure solution of $FeCl_3$, with no other complexing agents present, ascorbic acid has an opportunity to act unhindered to reduce Fe^{+++} iron to Fe^{++} iron. This explains why in the pure system with 46.5 mg of ascorbic acid (i.e. 5 moles of ascorbic acid to 1 mole of iron), practically all the iron (i.e. 3 mg of iron) became ionisable. Such a phenomenon was not observed in the complex meal system where the iron was already in a bound form and ascorbic acid can only act on the iron that is released from the food in a soluble form. As seen from Table 23, at this level of ascorbic acid (46.5 mg) in standard meal, only 1.6 mg out of the 3 mg has been released in a soluble form thus effectively curtailing the scope for ascorbic acid to excert its effect.

Another mode of action of ascorbic acid is by forming a soluble chelate with ferric form of iron at an acid pH, which remains stable and soluble even as the pH increases to alkalinity in the small intestine (Conrad and Schade, 1968). In this manner, ascorbic acid prevents the complexing of iron with other inhibitory ligands which react at the alkaline pH of the intestine to make it insoluble (Hurrell, 1984). This effect of ascorbic acid in the STD meal was evident in the soluble iron fraction which rose steadily from 39% at the lowest level of ascorbic acid (2.8mg) to 63.3% at 186mg.

It has also been hypothesised that ascorbic acid preferentially donates its iron to the brush border receptors at

the intestinal mucosa, thereby helping in an increased uptake of the metal by the mucosal cells. This mode of action of ascorbic acid, however can not be mimicked in the in vitro situation which could possibly be one of the reasons for a greater magnitude of increase observed with ascorbic acid in the in vivo situation as against in the vitro setting.

Citrate

Dose effect : The dose effect of citrate in the STD meal was different from that in the pure system. With only a $FeCl_3$ solution, increasing levels of citrate had no effect on iron availability. However, a moderate effect of making iron more available was evident in the STD meal when citrate was added in increasing dose levels from 30 mg to 960 mg resulting in nearly two fold increase (from 4.8% to 8.6%).

Similar increase in the in vitro % solubilization of iron from beans has been reported by Kojima et al (1981). The authors found that on addition of increasing doses of citrate (1m M to 20 mM) there was three fold increase in solubilization of iron (from 13% to 38%)

There are no in vivo studies available in the literature where such a dose effect of citrate has been investigated.

Interaction effect : In the present study, an interesting finding with respect to citrate was that, when added in combination with other enhancers and inhibitors it acted as a potent enhancer of iron availability, both in the pure FeCl3 as

well as in the STD meal. This indicated that in the presence of other constituents, especially ascorbic acid, citrate exerted on enhancing effect on iron availability in the in vitro situation.

These findings are consistent with the observations of Hazell and Johnson (1987) in the in vitro system. They reported that citrate (325 mg), when present along with asocrbic acid (32 mg) produced a greater enhancement (from 3.8% to 25%) than when it was present alone (from 3.8% to 16.7%). Their findings revealed that on an average, fruits and vegetables containing 627 mg citrate had 19 to 20% iron diffusability as against only 4% from those fruits and vegetables that did not contain appreciable quantities of citrate.

Similar observations have been made by Ballot et al (1987) in an in vivo human study. In this study, a combination of citrate and ascorbic acid, added to a rice meal at levels equivalent to that present in commercially available orange juice (750 mg and 33 mg respectively), resulted in a significant seven fold increase in iron absorption as against only four and a half increase brought about by addition of ascorbic acid per se (33mg) to the meal.

Mechanism of action : The observation that citrate had no effect on a pure $Fecl_3$ solution but produced on effect in the STD meal as well as in pure $FeCl_3$ in the presence of other constituents indicates a potentiating role of citric acid. It has been suggested by Reddy et al, (1986) that a combination of citrate, a chelating agent and ascorbic, acid a reducing agent, helps in enhancing iron mobilization to a greater extent than either of them present alone in the medium. While

citrate can chelate iron and form a low molecular weight polymer $\{(Fe - Cit)_2\ OH\}^3_n$ that remains soluble at the alkaline pH of the intestine (due to the coating of citrate on the citrate - iron chelate (Spiro et al, 1967a)), citrate does not possess the ability to reduce the ferric form of iron to ferrous form. This is accomplished by ascorbic acid which enhances the avaialbility of iron by producing Fe^{2+} from Fe^{3+} in the medium. In this manner, a combination of these two enhancers can result in a greater magnitude of enhancement in iron availability. These observations tally with the findings of the present study where citrate, though exhibited moderate enhancing effect when added alone to the STD meal, turned out to be a strong enhancer of iron availability when present in combination with ascorbic acid.

Tannate

Dose effect : Among the inhibitors tested in the present study, tannate exhibited a marked dose dependent inhibition, reducing iron availability from 21% to 2.6% in the pure system and from 4.8% to 3.8% in the standard meal. The inhibitory effect of tannate has been observed by several investigators in vitro (Narasinga Rao and Prabhavati, 1978 and 1982; Christian and Seshadri, 1989) and in vivo (Gillooly et al, 1983; Brune et al, 1989); although studies which have included dose effect are only a few.

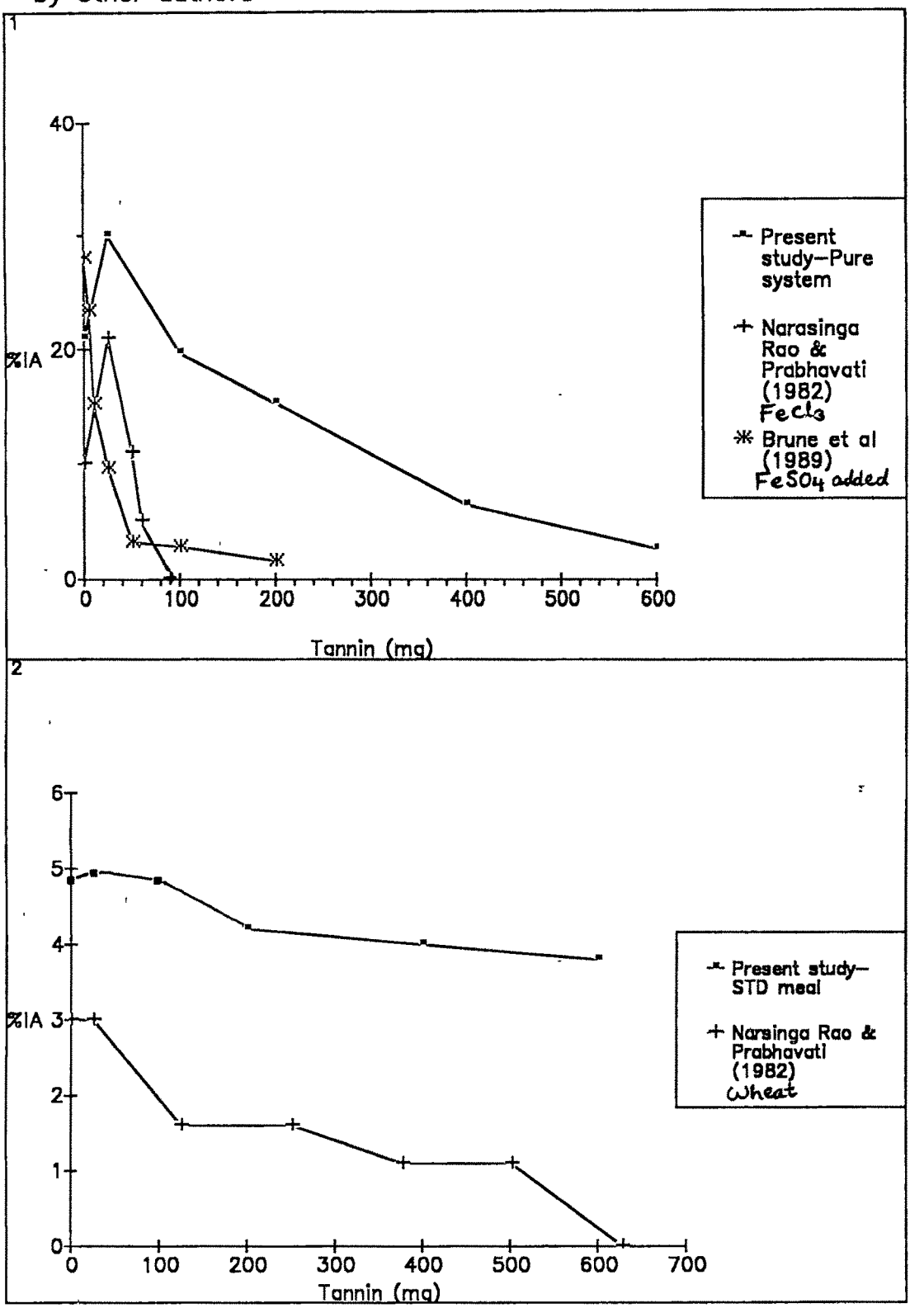
This trend of decreased iron availability from the pure system with increasing dose levels of tannate is similar to that reported in the in vitro system by Narasinga Rao and Prabhavati

(1982) (Figure 20). The authors observed that from a pure FeCl3 solution, there was no significant reduction in iron availability uptill the level of 57 mg tannin after which there was a progressive decrease in iron availability till all the iron in the solution was bound by 90 mg tannin. When the authors used wheat in place of FeCl3, however, the amount of tannate required to completely bind the iron was much higher (628 mg bound 6.9 mg iron in wheat resulting in negligible availability of iron). This trend was similar to the decrease observed in the present study where 600 mg tannate bound nearly 96% of the 3 mg iron present in the STD meal. However, on a molar ratio basis, the amount of tannate needed to bind iron in the meal tested in the present study was much greater (6.5 moles per mole of iron) as against that reported by Narasinga Rao and Prabhavati (1982) (three moles per mole of iron).

In the in vivo situation it has been reported by Brune et al (1989) that only 200 mg of tannate is required to reduce iron absorption from a wheat roll (to which 3.5 mg Fe was added as $FeSO_4$) from 28% to 1.6%, indicating 94% reduction in iron absorption. The magnitude of inhibition in their study is much greater than that observed in the in vitro setting in the present study, where 600 mg of tannate is required to bring about similar reduction (96 to 98%) in iron availability (Figure 20). Hence, as in the case of ascorbic acid, though the direction of effect and the shape of the dose effect curve with increasing levels of tannate are similar in the in vitro and in vivo situation, the magnitude

Figure 20

Comparison of dose effect of tannin on iron availability in the present study with that reported by other authors



of inhibitory effect for a given level of tannin is much higher in the in vivo setting.

Interaction effect : When tannate was studied for its interaction when present with the other food constituents in the present study (Phase III and V), it was found that it exerted a significant inhibitory effect on iron availability even in the presence of strong enhancers such as ascorbate and citrate in the medium. Reference to the literature reveals that there are no studies that have investigated the interaction effect of tannate with other constituents, barring some studies where the inhibitory effect of tea has been reported to be counteracted by ascorbate, as already discussed under the subhead 'ascorbic acid'.

Mechanism of action : The trend of decreased iron availability with tannate could be related to the precipitating property of polyphenols that bind with iron, resulting in the formation of an insoluble complex, thereby rendering the iron unavailable for absorption (Disler et al, 1975).

As a group, polyphenolic compounds comprise of polymerized molecules that contain aromatic rings with one or more hydroxyls attached to them as their func tional group. In general, tannins that are present in vegetable kingdom, are large polyphenols and depending upon their monomeric units, they are divided into two subgroups, condensed tannins and hydrolysable tannins. It has been reported that condensed tannins that contain flavnols (eg. catechin) as their monomers are not as inhibitory in nature as the hydrolysable tannins that contain gallic acid as their

monomer (Brune et al, 1989). The authors found that when equimolar amounts of catechin and gallic meal acid were (of wheat rolls and margarine), catechn did not affect iron absorption while gallic acid strongly inhibited iron absorption. Towards an explanation for this kind of observation, the authors suggested that the insolubility of the catechin molecule in acquous condition prevents a complex formation of this polyphenol with iron in the gastrointestinal lumen. Gallic acid on the other hand,readily reacts with iron resulting in a blackish precipitate which is insoluble in water. Hence, condensed tannins, as a group do not interfere with iron absorption while hydrolysable tannins probably release their reactive gallic acid groups at the acidic pH (of the stomach) through the process of hydrolysis, and these residuces, in turn,combine with iron at the alkaline pH (of the intestine), thereby rendering it unavailable for absorption.

This is the machanism by which tannate inhibits iron availability in the vitro system also. However, as discussed earlier, there seem to be a greater reduction in iron absorption in vivo than in vitro, with a certain level of tannate. The reasons for this kind of difference in the magnitude of effect of tannate in the two settings are obscure at present. But the results indicate that in vitro, the effect of tannin is different in magnitude from that in vivo.

Calcium Phosphate

Dose effect : The other inhibitor that exhibited a significant depressing effect on iron avilability in the present

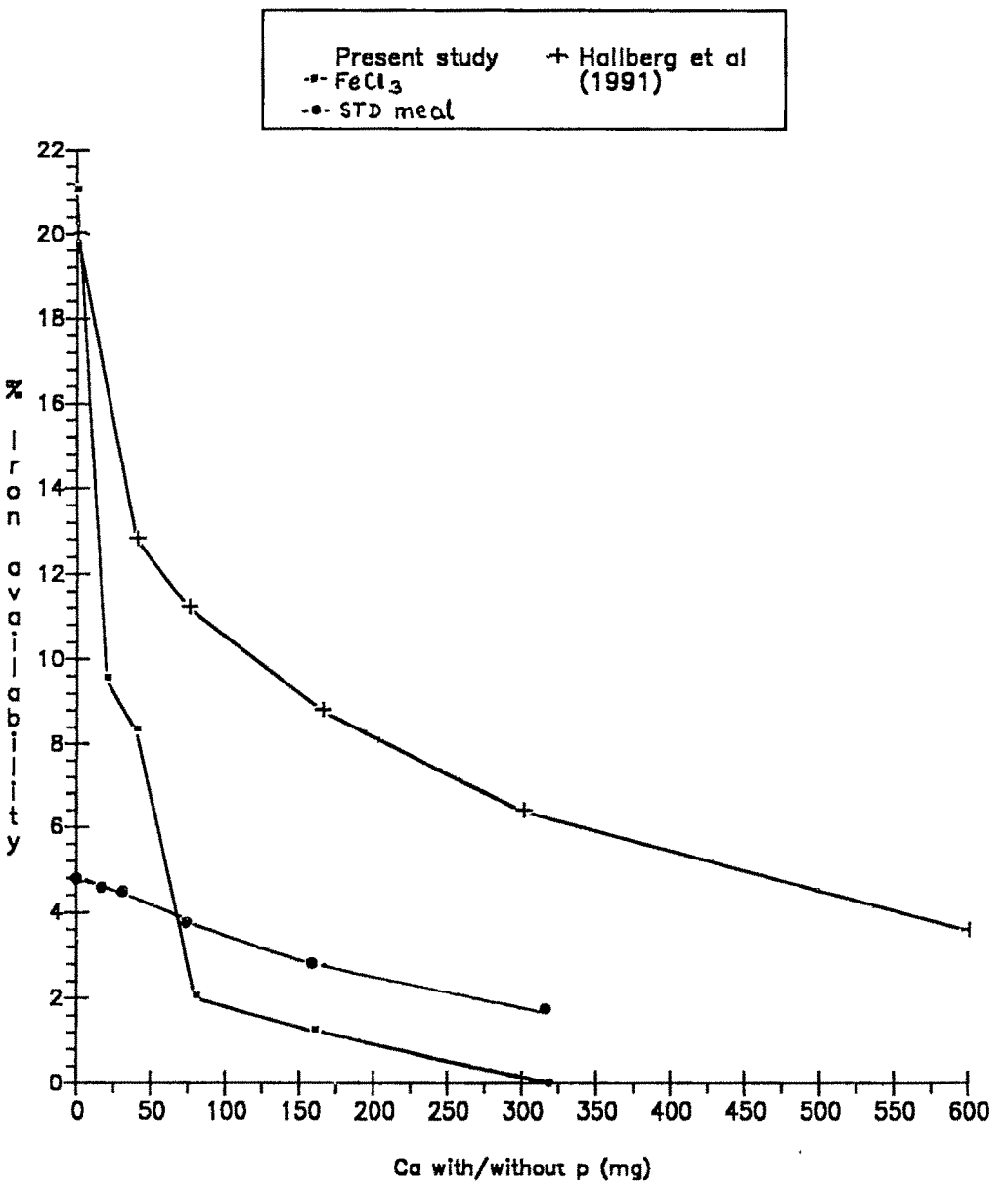
study was calcium phosphate. Its inhibiting effect was in fact stronger than that observed with tannate. Addition of increasing doses of calcium phosphate in the ratio of 1:2 resulted in negligible iron availability in the pure system (from 21% to 0%). In the STD meal too, almost 2.9 mg iron (out of 3 mg) was bound to calcium phosphate at the highest dose level.

Since there are no in vitro studies available for comparision, the trend of reduction in iron availability on addition of calcium phosphate in the present study, is compared with the in vivo effect of increasing doses of calcium, as reported by Hallberg et al (1991) (Figure 21). In this human study, increasing doses of calcium (as $CaCl_2$) resulted in a dose dependent reduction in iron absorption from a wheat bun, to which 3.5 mg iron was added as $FeSO_4$. It should be noted, that the magnitude of reduction in iron absorption brought about by 300 mg calcium per se in Hallberg's study was lower (70%) than that observed with a similar quantity of calcium (320 mg) and 640 mg phosphate added together to the pure system in the present study (100% reduction). This could be explained by the observations of Monsen and Cook (1976) who reported that addition of calcium and phosphate together (202: 414 mg respectively) to a semi-synthetic meal resulted in a significantly higher reduction (73%) in iron absorption than either of the two added alone to the meal (32% in each case).

When the dose effect of calcium and phosphate in the standard meal system was evaluated in the present study, it was revealed that there was a maximum reduction of 52% in iron availability

Figure 21

Comparison of dose effect of calcium and phosphate added to $FeCl_3$/STD meal in the present study with that of calcium per se, reported by Hallberg et al (1991).



(from 4.8% to 2.3%) with the highest dose level of calcium and phosphate. Similar degree of inhibition has been reported by Dawson-Hughes et al (1986) in an in vivo human study. The authors found 57% reduction in iron absorption when when 500 mg calcium (as calcium carbonate) was added to a standard meal. When the same amount of calcium was fed as hydroxyapatite to the subjects, there was 54% reduction in iron absorption. Similar findings have been reported by Cook et al (1991) where addition of calcium supplements (600 mg) as calcium carbonate, calcium citrate or calcium phosphate to a low bioavailability breakfast meal resulted in 42 to 63% reduction in iron absorption, with a pooled mean of 55%. The authors stated, however, that the greatest inhibition was observed with calcium phosphate. These findings are in line with the resuslts of the present study, where presence of both these constituents together resulted in a significant inhibition of iron availability.

Interaction effect : When all the selected food constituents were added together to the pure system or STD meal, calcium phosphate emerged as a strong inhibitor of iron availability, its inhibitory effect being stronger than that of tannate. There are no in vitro studies available in the literature that have investigated such an interaction effect of calcium-phosphate.

In the in vivo human study of Hallberg et al (1991), when the authors investigated the effect of interaction of calcium and phytate on iron absorption, they observed that addition of calcium chloride (600 mg Ca) to the dough, while baking the

rolls, reduced the degradation of endogenous phytate on baking, thereby resulting in a greater inhibition (80%) of iron absorption than that observed when calcium was present along with phytate in the flour. The resultant phytate content of the bread increases to levels that interfered with iron absorption. Calcium addition, after baking resulted in less inhibition, suggesting that the calcium acted on its own as well as in interaction with phytate to reduce iron absorption.

Mechanism of action : It is possible that in the alkaline environment of the intestine, calcium and phosphate together bind with iron, forming an insoluble complex and thereby render it unavailable for absorption (Monsen and Cook, 1976). Barton et al (1983) have suggested however, that calcium seems to reduce absorptin of iron by competing, partially, for iron receptor sites at the intestinal mucosal level, and may also influence adversely, the transfer of iron into the systemic circulation. This has been restated by Menhansho et al (1987) who demonstrated on the basis of a number of short and long term, isolated duodenal loop experiments in rats, that it is the transfer of iron to the serosal side which is disrupted by the presence of calcium in the medium. Such an effect however, cannot be simulated in the in vitro situation. Thus, in the in vitro situation only the formation of insoluble complexes is a plausible explanation for the inhibitory effect of calcium and phosphate.

Phytate

Dose effect : One of the variables that did not show the expected inhibitory effect in the present study was phytate when added in increasing doses in a pure chemical form to the pure system or to the STD meal. This consitituent increased rather than decreased iron availability in the pure system (from 21% to 50%) and in the STD meal (from 4.8% to 6.5%). An interesting trend seen in the STD meal on addition of increasing levels of phytate was that at the first two levels, there was a decrease in iron availability (Table 22).

In contrast to the other constituents, where a consistently enhancing (ascorbate, citrate) or inhibiting effect (tannate, calcium and phosphate) has been reported in the literature, from in vitro as well as in vivo studies with respect to the effect of phytate, studies have yielded somewhat conflicting results. Though the in vitro stuies of Narasinga Rao and Prabhavati (1978) and Hazell and Johnson (1987) have indicated an inhibitory effect of pure Na-phytate, some animal studies have reported an enhancing effect of phytate (Gordon and Chao, 1984). In this rat study, phytate when added in a pure chemical form to the diet (0.66%) significantly increased the relative biological value from 100% (with $FeSO_4$) to 124% (with Na-phytate). This was similar to the effect of pure form of phytate observed in the present study.

Some of the in vivo human studies have shown that phytate per se does not cause an inhibition of iron absorption (Hurrell et al, 1988). In this study, it was found that soy isolates that

have been claimed to have the maximum degree of inhibition (relative iron absorption of 10% as compared to 100% from reference egg white meal) did not increase the iron absorption in human subjects on removal of 90% phytate (Hurrell et al, 1988). Further, the inhibitory effect of bran that has been attributed to phytate in many in vivo human studies (Morris and Ellis, 1982; Hallberg et al, 1987) does not completely revert back to the basal level on dephytinising the bran. In the more recent human studies, however, addition of pure Na-phytate has been reported to inhibit iron absorption significantly, which is essentially does related (Hallberg et al, 1989). In view of the varying reports on the role of phytate on iron absorption, it has been suggested that presence of some other factors along with phytate may explain the inhibitory effect of bran on iron absorption rather than presence of phytate alone (Hallberg et al, 1987). Some of the possible factors are fibre, polyphenols and certain metals such as calcium, zinc, phosphorus etc (Morris, 1983). In the present in vitro study, phytate at the levels tested did not reproduce the effects seen in other in vitro studies or in the in vivo ones, an explanation for which may be sought in the proposed mechanism of action of phytate.

Mechanism of action : It has been suggested that it is the proportion of phytate to iron in the reacting medium which influences the solubility of the phytate - iron complex (Young, 1936). It has been reported by the above author that when iron is present in excess of phytate, it completely precipitates phytate (1 mg iron combining with 1 mg phytin-P) in the form of

di-or tetra-ferric phytate complexes. These complexes have been reported to be relatively insoluble than mono-ferric-phytate, which is highly bioavailable to both animals and humans (Morris and Ellis, 1982). Hence it could be possible that in the present study where phytate was present in excess of iron (more than one to 28 moles per mole of iron), it formed a soluble complex with iron, thereby increasing the iron availability in the vitro system. It should be noted however, that when phytate was added to the pure system, in the form of wheat bran or rice bran there was a significant reduction in iron availability (from 21% to 1.9%), a finding consistent with the in vivo studies on the effect of bran (Morris and Ellis, 1982; Hallberg et al, 1987). Thus, the in vitro technique used in the present study reproduced reasonably well the in vivo effect of bran, as well as phytate present natively in foods but was very different with pure phytate.

Oxalate

Dose effect : Oxalate which is believed to be inhibitory in nature, also resulted in an increased availability of iron in the present study (from 21% to 46% in the pure system and from 4.8% to 6.4% in the STD meal). In the STD meal however, there was an initial reduction in iron availability, a trend similar to that observed with phytate in the present study.

There are no in vitro studies that have investigated the effect of oxalate per se on iron availability. However, addition of green leafy vegetables such as spinach has been shown to

result in a significant depressing effect (80% reduction) on iron availability (Kojima et al, 1981). It was suggested by the authors that since spinach is rich in oxalate and fibre, these constituents may be responsible, partially for the inhibitory effect of this GLV on iron availability. In the in vitro studies carried out in our Department (Chawla et al, 1988), a trend of decreased iron availability was observed with the GLVs containing large amounts of oxalate (6 to 7 g/kg) though no significant correlation was found between the two.

In the in vivo system, studies using a rat model have either shown no inhibition on addition of spinach (Van campen and Welch, 1980) or an enhancing effect of oxalate, added to the diet, in amounts equivalent to that present in spinach (2.1%) (Gordon and Chao, 1984). In the only in vivo human study available where pure oxalate (1g) was added to cabbage, there was a moderate inhibitory effect on iron absorption (Geometric mean reducing from 0.32 mg to 0.29% mg) (Gillooly et al, 1983).

Mechanism of action : The observation that oxalate decreased iron availablity initially, when added to the STD meal in the present study, may be explained by the suggestion of Lee (1982). According to him oxalate may vary in its effect on iron availability depending upon the strength and stablility of the chelate that it forms with iron. It is possible that at lower levels oxalate may form stable insoluble chelates with iron resulting in a decreased iron availability while at higher dose levels, the strength of the chelate formed may be low thereby

dissociating easily in the medium and resulting in an increased iron availability. Presence of other ligands in the medium such as calcium, ascorbic acid, citrate, fibre etc. may also excert some effect on the net availability of iron. It is possible that the inhibitory effect of certain green leafy vegetables may be the resultant effect of interaction of all these factors, present together in the medium, rather than the oxalate content per se.

In essence, the direction of the trends observed in the in vitro iron avaialbility, on addition of four of the six constituents studied in the pure system or STD meal were consistent with those reported in the in vivo human studies in the literature, while that of phytate and oxalate was very different. Hence, for evaluating the predictive model, evolved in the present study, only these four variables (i.e. ascorbate, citrate, tannate and calcium phosphate) were incorported in the equation.

Evaluation of the predictive models (Phase VI)

On the basis of the interaction effect of various food constituents on iron availability, two predictions equations were evolved in the present study, from the pure system and the standard meal respectively. Evaluation of these two equations and the model of Monsen and Balintfy (1982) led to two major findings. Firstly, the standard meal equation, which was evolved on the basis of four food constituents, namely, ascorbic acid, citric acid, tannate and calcium phosphate, showing expected trends with iron avaialbility, was found to have a better

predictive power (r=0.76) than that evolved from the pure system (r=0.59). Despite the observation that the correlation obtained with the pure system equation was 0.59, there were some serious limitations in using this equation for predicting iron availablity from complex meals. One of the limitations was that the computed values for iron availability were higher when the pure system equation was used than the STD meal equation, in comparison to the estimated values values for iron availability. The regression constant in the pure system equation was much higher (20.9) than that in the STD meal equation (4.28), resulting in a gross overestimate of % iron availability, when the pure system equation was applied to the complex meal system.

Another serious limitation was that the amount of enhancers or inhibitors required to bring about a certain degree of enhancement/inhibition in iron availability in the pure system was very different than that required in the STD meal system, resulting in regression coefficients that were different for all the four constituents, when compared to the STD meal equation (Table 28).

The second finding was, that when the model of Monsen and Balintfy (1982), that incorporated only enhancers of iron availability was applied to the typical Indian vegetarian meals, it exhibited a low predictive power (r=0.19). This was not surprising since the presence of large quantities of inhibitors, in the cereal based Indian meals, could have a greater influence on the net availability of iron than the limited number of enhancers present in them (none of the meals contained animal

tissue). It would be appropriate, therefore, to suggest that the equation evolved in the present study is more applicable to meals that contain larger quantities of inhibitors than enhancers, such as those habitually consumed in India and South East Asia. The model of Monsen and Balintfy (1982), could provide a more practical quantitative estimate of iron bioavailability from meals that contain appreciable quantities of enhancers and less inhibitors, such as those consumed in Western countries.

In conclusion, the findings of the present study supported the hypothesis that indeed, a regression equation that integrated the effects of both enhancers and inhibitors, could predict availability of iron from Indian vegetarian meals better, than the one that incorporated only the enhancers. However, the hypothesis that an equation evolved on the basis of the pure system would predict with reasonable accuracy the iron availability from complex meals was rejected in view of the limitations of using the pure system equation, as discussed earlier.

Limitations of the STD meal equation

There are certain limitations of the model evolved in the present study. Since the basis of this model was an in vitro technique, which dose not take into account the humoral and mucosal factors, the values obtained can only be termed as relative, rather than absolute indicators of what would actually happen in the in vivo situation. Further, certain food constituents such as ascorbic acid and tannate differ in the

magnitude of their effect on iron availability in the in vitro vs in vivo situation. However, in view of the fact the effect of both these constituents is opposite in direction (ascorbic acid being an enhancer and tannate being an inhibitor), their net effect on the predicted iron availability may not be very different from that observed in the in vivo system. Therefore, despite these limitations, the present model may be able to provide a reasonably good quantitative estimate of iron availability from a given meal of known composition.

Practical implications of the study

The practical implications of an updated model, evolved in the present study are, to provide a quick, quantitative assessment of the amount of nonheme iron likely to be absorbed from a given cereal based meal of known compostion. This would help in classifying various meals into low medium and high bioavailability meals. Moreover, when the concentration of enhancers and inhibitors are known in a meal, the equation can also be used to predict the amount of enhancers, required to achieve a desired level of iron availability from the same meal.

For example, a typical Indian breakfast meal consisting of wheat paratha, vegetable and tea (in amounts equivalent to those contained in Meal no.1, Phase I) will contain negligible ascorbic acid, 127.5 mg citric acid, 127 mg tannic acid, 98 mg calcium and 246 mg phosphorus. The predicted iron availability from this meal would be 3.6% (by incorporating these values in the STD meal equation). In order to increase the availability of iron to

nearly twice this value, one could increase the amount of enhancers in the meal and/or decrease the amount of inhibitors. One way to do this is to add a fruit which is rich in ascorbic acid and citric acid such as orange. If 100g orange is added to the meal, it would provide an additional 50 mg ascorbic acid and 980mg citric acid, resulting in a net increase of 2.6% in iron availability. Hence the predicted value would increase from 3.6% to 6.2% (nearly double the basal value). Such practical suggestions would assist in designing meals that tend to maximise availability of dietary iron. This would not only help in paving the way for better iron status of individuals, but would also assist in providing effective and more practical nutrition counselling in various community health programmes, hospitals and other health care centres.